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(54) Title: LIGANDS FOR PHOSPHATASE BINDING ASSAY

(57) Abstract

Disclosed are new ligands for use in a binding assay for proteases and phosphatases, which contain cysteine in their binding sites or as a necessary structural component for enzymatic binding. The sulfhydryl group of cysteine is the nucleophilic group in the enzyme's mechanistic proteolytic and hydrolytic properties. The assay can be used to determine the ability of new, unknown ligands and mixtures of compounds to competitively bind with the enzyme versus a known binding agent for the enzyme, e.g., a known enzyme inhibitor. By the use of a mutant form of the natural or native wild-type enzyme, in which serine, or another amino acid, e.g., alanine, replaces cysteine, the problem of interference from extraneous oxidizing and alkylating agents in the assay procedure is overcome. The interference arises because of oxidation or alkylation of the sulfhydryl, -SH (or $-S^-$), in the cysteine, which then adversely affects the binding ability of the enzyme. Specifically disclosed is an assay for tyrosine phosphatases and cysteine proteases, including capsases and cathepsins, e.g., Cathepsin K(O2), utilizing scintillation proximity assay (SPA) technology. The assay has important applications in the discovery of compounds for the treatment and study of, for example, diabetes, immunosuppression, cancer, Alzheimer's disease and osteoporosis. The novel feature of the use of a mutant enzyme can be extended to its use in a wide variety of conventional colorimetric, photometric, spectrophotometric, radioimmunoassay and ligand-binding competitive assays.

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TITLE OF THE INVENTION
LIGANDS FOR PHOSPHATASE BINDING ASSAY

FIELD OF THE INVENTION

This invention relates to the use of mutant phosphatase and protease enzymes in a competitive binding assay. Specific examples are the enzymes, tyrosine phosphatase and cysteine protease, e.g. Cathepsin K, and the assay specifically described is a scintillation proximity assay using a radioactive inhibitor to induce scintillation.

BACKGROUND OF THE INVENTION

The use of the scintillation proximity assay (SPA) to study enzyme binding and interactions is a new type of radioimmunoassay and is well known in the art. The advantage of SPA technology over more conventional radioimmunoassay or ligand-binding assays, is that it eliminates the need to separate unbound ligand from bound ligand prior to ligand measurement. See for example, *Nature,* Vol, 341, pp. 167-178 entitled "Scintillation Proximity Assay " by N. Bosworth and P. Towers, *Anal. Biochem.* Vol. 217, pp. 139-147 (1994) entitled "Biotinylated and Cysteine-Modified Peptides as Useful Reagents For Studying the Inhibition of Cathepsin G" by A.M. Brown, et al., *Anal. Biochem.* Vol. 223, pp. 259-265 (1994) entitled "Direct Measurement of the Binding of RAS to Neurofibromin Using Scintillation Proximity Assay" by R. H. Skinner *et al.* and *Anal. Biochem.* Vol. 230, pp. 101-107(1995) entitled "Scintillation Proximity Assay to Measure Binding of Soluble

Fibronectin to Antibody-Captured alpha5ß1 Integrin" by J. A. Pachter *et al.*

The basic principle of the assay lies in the use of a solid support containing a scintillation agent, wherein a target enzyme is attached to the support through, e.g., a second enzyme-antienzyme linkage. A known tritiated or I^{125} iodinated binding agent, i.e., radioligand inhibitor ligand for the target enzyme is utilized as a control, which when bound to the active site in the target enzyme, is in close proximity to the scintillation agent to induce a scintillation signal, e.g., photon emission, which can be measured by conventional scintillation/radiographic techniques. The unbound tritiated (hot) ligand is too far removed from the scintillation agent to cause an interfering measurable scintillation signal and therefore does not need to be separated, e.g., filtration, as in conventional ligand-binding assays.

The binding of an unknown or potential new ligand (cold, being non-radioactive) can then be determined in a competitive assay versus the known radioligand, by measuring the resulting change in the scintillation signal which will significantly decrease when the unknown ligand also possesses good binding properties.

However, a problem arises when utilizing a target enzyme containing a cysteine group, having a free thiol linkage, -SH,(or present as $-S^-$) which is in the active site region or is closely associated with the active site and is important for enzyme-ligand binding. If the unknown ligand or mixture, e.g. natural product extracts, human body fluids, cellular fluids, etc. contain reagents which can alkylate, oxidize or chemically interfere with the cysteine thiol group such that normal enzyme-ligand binding is disrupted, then false readings will occur in the assay.

What is needed in the art is a method to circumvent and avoid the problem of cysteine interference in the scintillation proximity assay (SPA) procedure in enzyme binding studies.

SUMMARY OF THE INVENTION

We have discovered that by substituting serine for cysteine in a target enzyme, where the cysteine plays an active role in the wild-type enzyme-natural ligand binding process, usually as the catalytic nucleophile in the active binding site, a mutant is formed which can be successfully employed in a scintillation proximity assay without any active site cysteine interference.

This discovery can be utilized for any enzyme which contains cysteine groups important or essential for binding and/or catalytic activity as proteases or hydrolases and includes phosphatases, e.g., tyrosine phosphatases and proteases, e.g. cysteine proteases, including the cathepsins, i.e., Cathepsin K (O2) and the capsases.

Further, use of the mutant enzyme is not limited to the scintillation proximity assay, but can be used in a wide variety of known assays including colorimetric, spectrophotometric, ligand-binding assays, radioimmunoassays and the like.

We have furthermore discovered a new method of amplifying the effect of a binding agent ligand, e.g., radioactive inhibitor, useful in the assay by replacing two or more phosphotyrosine residues with 4-phosphono(difluoromethyl) phenylalanine (F_2Pmp) moieties. The resulting inhibitor exhibits a greater and more hydrolytically stable binding affinity for the target enzyme and a stronger scintillation signal.

By this invention there is provided a process for determining the binding ability of a ligand to a cysteine-containing wild-type enzyme comprising the steps of:

(a) contacting a complex with the ligand, the complex comprising a mutant form of the wild-type enzyme, in which cysteine, at the active site, is replaced with serine, in the presence of a known binding agent for the mutant enzyme, wherein the binding agent is capable of binding with the mutant enzyme to produce a measurable signal.

Further provided is a process for determining the binding ability of a ligand, preferably a non-radioactive (cold) ligand, to an active site cysteine-containing wild-type tyrosine phosphatase comprising the steps of:

(a) contacting a complex with the ligand, the complex comprising a mutant form of the wild-type enzyme, the mutant enzyme being PTP1B, containing the same amino acid sequence 1-320 as the wild type enzyme,except at position 215, in which cysteine is replaced with serine in the mutant enzyme, in the presence of a known radioligand binding agent for the mutant enzyme, wherein the binding agent is capable of binding with the mutant enzyme to produce a measurable beta radiation-induced scintillation signal.

Also provided is a new class of peptide binding agents selected from the group consisting of:
N-Benzoyl-L-glutamyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanineamide (BzN-EJJ-$CONH_2$), where E is glutamic acid and J is 4-phosphono(difluoro-methyl)]-L-phenylalanyl;
N-Benzoyl-L-glutamyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide;
N-Acetyl-L-glutamyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide;
L-Glutamyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono-(difluoromethyl)]-L-phenylalanine amide;
L-Lysinyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono-(difluoromethyl)]-L-phenylalanine amide;
L-Serinyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono-(difluoromethyl)]-L-phenylalanine amide;
L-Prolinyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono-(difluoromethyl)]-L-phenylalanine amide; and
L-Isoleucinyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono-(difluoromethyl)]-L-phenylalanine amide; and their tritiated and I^{125} iodinated derivatives.

Further provided is a novel tritiated peptide, tritiated BzN-EJJ-$CONH_2$, being N-(3,5-Ditritio)benzoyl-L-glutamyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono-(difluoromethyl)]-L-phenylalanineamide, wherein E as used herein is glutamic acid and J, as used herein, is the (F_2Pmp) moiety, (4-phosphono(difluoromethyl)-phenylalanyl).

Furthermore there is provided a process for increasing the binding affinity of a ligand for a tyrosine phosphatase or cysteine protease comprising introducing into the ligand two or more 4-phosphono(difluoromethyl)-phenylalanine groups; also provided is the resulting disubstituted ligand.

In addition there is provided a complex comprised of:

(a) a mutant form of a wild-type enzyme, in which cysteine, necessary for activity in the active site, is replaced with serine and is attached to:

(b) a solid support.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 illustrates the main elements of the invention including the scintillation agent 1, the supporting (fluomicrosphere) bead 5, the surface binding Protein A 10, the linking anti-GST enzyme 15, the fused enzyme construct 20, the GST enzyme 25, the mutant enzyme 30, the tritiated peptide inhibitor 35, the beta radiation emission 40 from the radioactive peptide inhibitor 35 and the emitted light 45 from the induced scintillation.

FIGURE 2 (A and B) illustrates the DNA and amino acid sequences for PTP1B tyrosine phosphatase enzyme, truncated to amino acid positions 1-320. (Active site cysteine at position 215 is in bold and underlined).

FIGURE 3 (A, B and C) illustrates the DNA and amino acid sequences for Cathepsin K. The upper nucleotide sequence represents the cathepsin K cDNA sequence which encodes the cathepsin K preproenzyme (indicated by the corresponding three letter amino acid codes). Numbering indicates the cDNA nucleotide

position. The underlined amino acid is the active site Cys^{139} residue that was mutated to either Ser or Ala.

FIGURE 4 (A and B) illustrates the DNA and amino acid sequences for the capsase, apopain. The upper nucleotide sequence represents the apopain (CPP32) cDNA sequence which encodes the apopain proenzyme (indicated by the corresponding three letter amino acid codes). Numbering indicates the cDNA nucleotide position. The underlined amino acid is the active site Cys^{163} residue that was mutated to Ser.

DETAILED DESCRIPTION OF THE INVENTION

The theory underlying the main embodiment of the invention can be readily seen and understood by reference to FIGURE 1.

Scintillation agent 1 is incorporated into small (yttrium silicate or PVT fluomicro-spheres, AMERSHAM) beads 5 that contain on their surface immunosorbent protein A 10. The protein A coated bead 5 binds the GST fused enzyme construct 20, containing GST enzyme 25 and PTP1B mutant enzyme 30, via anti-GST enzyme antibody 15. When the radioactive e.g., tritiated, peptide 35 is bound to the mutant phosphatase enzyme 30, it is in close enough proximity to the bead 5 for its beta emission 40 (or Auger electron emission in the case of I^{125}) to stimulate the scintillation agent 1 to emit light (photon emission) 45. This light 45 is measured as counts in a beta plate counter. When the tritiated peptide 35 is unbound it is too distant from the scintillation agent 1 and the energy is dissipated before reaching the bead 5, resulting in low measured counts. Non-radioactive ligands which compete with the tritiated peptide 35 for the same binding site on the mutant phosphatase enzyme 30 will remove and/or replace the tritiated peptide 35 from the mutant enzyme 30 resulting in lower counts from the uncompeted peptide control. By varying the concentration of the unknown ligand and measuring the resulting lower counts, the inhibition at 50%(IC_{50}) for ligand binding to the mutant enzyme 30 can be obtained. This then is a measure of

the binding ability of the ligand to the mutant enzyme and the wild-type enzyme.

The term "complex" as used herein refers to the assembly containing the mutant enzyme. In its simplest embodiment, the complex is a solid support with the mutant enzyme attached to the surface of the support. A linker can also be employed. As illustrated in FIGURE 1, the complex can further comprise a bead (fluopolymer), anti-enzyme GST/enzyme GST-mutant enzyme-PTP1 linking construct, immunosorbent protein A, and scintillation agent. In general, the complex requires a solid support (beads, immunoassay column of e.g., Al_2O_3, or silica gel) to which the mutant enzyme can be anchored or tethered by attachment through a suitable linker, e.g., an immunosorbent (e.g, Protein A, Protein G, anti-mouse, anti-rabbit, anti-sheep) and a linking assembly, including an enzyme/anti-enzyme construct attached to the solid support.

The term "cysteine-containing wild-type enzyme",as used herein, includes all native or natural enzymes, e.g., phosphatases, cysteine proteases, which contain cysteine in the active site as the active nucleophile, or contain cysteine clearly associated with the active site that is important in binding activity.

The term "binding agent" as used herein includes all ligands (compounds) which are known to be able to bind with the wild-type enzyme and usually act as enzyme inhibitors. The binding agent carries a signal producing agent , e.g., radionuclide, to initiate the measurable signal. In the SPA assay the binding agent is a radioligand.

The term "measurable signal" as used herein includes any type of generated signal, e.g., radioactive, colorimetric, photometric, spectrophotometric, scintillation, which is produced when binding of the radioligand binding agent to the mutant enzyme.

The present invention assay further overcomes problems encountered in the past, where compounds were evaluated by their ability to affect the reaction rate of the enzyme in the phosphatase activity assay. However this did not give direct evidence that compounds were actually binding at the active site of the enzyme. The herein described invention binding assay using a substrate

analog can determine directly whether the mixtures of natural products can irreversibly modify the active site cysteine in the target enzyme resulting in inhibition of the enzymatic activity. To overcome inhibition by these contaminates in the phosphatase assay, a mutated Cys(215) to Ser(215) form of the tyrosine phosphatase PTP1B was cloned and expressed resulting in a catalytically inactive enzyme. In general, replacement of cysteine by serine will lead to a catalytically inactive or substantially reduced activity mutant enzyme.

PTP1B is the first protein tyrosine phosphatase to be purified to near homogeneity {Tonks *et al. JBC* 263, 6731-6737 (1988)} and sequenced by Charbonneau *et al. PNAS* 85, 7182-7186 (1988). The sequence of the enzyme showed substantial homology to a duplicated domain of an abundant protein present in hematopoietic cells variously referred to as LCA or CD45. This protein was shown to possess tyrosine phosphatase activity {Tonks *et al. Biochemistry* 27, 8695-8701 (1988)}. Protein tyrosine phosphatases have been known to be sensitive to thiol oxidizing agents and alignment of the sequence of PTP1B with subsequently cloned Drosophila and mammalian tyrosine phosphatases pointed to the conservation of a Cysteine residue {(M. Strueli *et al. Proc. Nat'l Acad USA*, Vol. 86, pp. 8698-7602 (1989)} which when mutated to Ser inactivated the catalytic activity of the enzymes. Guan *et al.*(1991) {J.B.C. Vol. 266, 17926-17030, *1991*} cloned the rat homologue of PTP1B, expressed a truncated version of the protein in bacteria, purified and showed the Cys at position 215 is the active site residue. Mutation of the Cys^{215} to Ser^{215} resulted in loss of catalytic activity. Human PTP1B was cloned by Chernoff *et al.* Proc. Natl. Acad. Sci. USA 87, 2735-2739 (1990).

Work leading up to the development of the substrate analog BzN-EJJ-$CONH_2$ for PTP1B was published by T. Burke *et al. Biochem. Biophys. Res. Comm.* 205, pp. 129-134 (1994) with the synthesis of the hexamer peptide containing the phosphotyrosyl mimetic F_2Pmp. We have incorporated the (F_2Pmp) moiety (4-phosphono-(difluoromethyl)phenylalanyl) into various peptides that led to the discovery of BzN-EJJ-$CONH_2$, (where E is glutamic acid and J as used herein is the F_2Pmp moiety) an active (5 nM) inhibitor

of PTP1B. This was subsequently tritiated giving the radioactive substrate analog required for the binding assay.

The mutated enzyme, as the truncated version, containing amino acids 1-320 (see FIGURE 2), has been demonstrated to bind the substrate analog Bz-NEJJ-$CONH_2$ with high affinity for the first time. The mutated enzyme is less sensitive to oxidizing agents than the wild-type enzyme and provides an opportunity to identify novel inhibitors for this family of enzymes. The use of a mutated enzyme to eliminate interfering contaminates during drug screening is not restricted to the tyrosine phosphatases and can be used for other enzyme binding assays as well.

Other binding assays exist in the art in which the basic principle of this invention can be utilized, namely, using a mutant enzyme in which an important and reactive cysteine important for activity can modified to serine (or a less reactive amino acid) and render the enzyme more stable to cysteine modifying reagents, such as alkylating and oxidizing agents. These other ligand-binding assays include, for example, colorimetric and spectrophotometric assays, e.g. measurement of produced color or fluorescence, phosphorescence (e.g. ELISA, solid absorbant assays) and other radioimmunoassays in which short or long wave light radiation is produced, including ultraviolet and gamma radiation).

Further, the scintillation proximity assay can also be practiced without the fluopolymer support beads (AMERSHAM) as illustrated in FIGURE 1. For example, Scintistrips® are commercially available (Wallac Oy, Finland) and can also be employed as the scintillant-containing solid support for the mutant enzyme complex as well as other solid supports which are conventional in the art.

The invention assay described herein is applicable to a variety of cysteine-containing enzymes including protein phosphatases, proteases, lipases, hydrolases, and the like.

The cysteine to serine transformation in the target enzyme can readily be accomplished by analogous use of the molecular cloning technique for Cys^{215} to Ser^{215} described in the below-cited reference by M. Strueli *et al.*, for PTP1B and is hereby incorporated by reference for this particular purpose.

A particularly useful class of phosphatases is the tyrosine phosphatases since they are important in cell function. Examples of this class are: PTP1B, LCA, LAR, DLAR, DPTP(See Strueli et al., below). Ligands discovered by this assay using, for example, PTP1B can be useful, for example, in the treatment of diabetes and immunosuppression.

A useful species is PTP1B, described in *Proc. Nat'l Acad USA*, Vol. 86, pp. 8698-7602 by M. Strueli *et al.* and *Proc. Nat'l Acad Sci. USA*, Vol 87, pp. 2735-2739 by J. Chernoff *et al.*

Another useful class of enzymes is the proteases, including cysteine proteases (thiol proteases), cathepsins and capsases.

The cathepsin class of cysteine proteases is important since Cathepsin K (also termed Cathepsin O2, see *Biol. Chem. Hoppe-Seyler*, Vol. 376 pp. 379-384, June 1995 by D. Bromme *et al.*) is primarily expressed in human osteoclasts and therefore this invention assay is useful in the study and treatment of osteoporosis. See US Patent 5,501,969 (1996) to Human Genome Sciences for the sequence, cloning and isolation of Cathepsin K (O2). See also *J. Biol. Chem.* Vol. 271, No. 21, pp. 12511-12516 (1996) by F. Drake *et al.* and *Biol. Chem. Hoppe-Seyler*, Vol. 376, pp. 379-384(1985) by D. Bromme *et al., supra.*

Examples of the cathepsins include Cathepsin B, Cathepsin G, Cathepsin J, Cathepsin K(O2), Cathesin L, Cathepsin M, Cathepsin S.

The capsase family of cysteine proteases are other examples where the SPA technology and the use of mutated enzymes can be used to determine the ability of unknown compounds and mixtures of compounds to compete with a radioactive inhibitor of the enzyme. An active site mutant of Human Apopain CPP32 (capsase-3) has been prepared. The active site thiol mutated enzymes are less sensitive to oxidizing agents and provide an opportunity to identify novel inhibitors for this family of enzymes.

Examples of the capsase family include: capsase-1(ICE), capsase-2 (ICH-1), capsase-3 (CPP32, human apopain, Yama), capsase-4(ICE_{rel}-11, TX, ICH-2), capsase-5(ICE_{rel}-111, TY), capsase-

6(Mch2), capsase-7(Mch3, ICE-LAP3, CMH-1), capsase-8(FLICE, MACH, Mch5), capsase-9 (ICE-LAP6, Mch6) and capsase-10(Mch4).

Substitution of the cysteine by serine (or by any other amino acid which lowers the activity to oxidizing and alkylating agents, e.g., alanine) does not alter the binding ability of the mutant enzyme to natural ligands. The degree of binding, i.e., binding constant, may be increased or decreased. The catalytic activity of the mutant enzyme will, however, be substantially decreased or even completely eliminated. Thus, natural and synthetic ligands which bind to the natural wild-type enzyme will also bind to the mutant enzyme.

Substitution by serine for cysteine also leads to the mutant enzyme which has the same qualititative binding ability as the natural enzyme but is significantly reduced in catalytically activity. Thus, this invention assay is actually measuring the true binding ability of the test ligand.

The test ligand described herein is a new ligand potentially useful in drug screening purposes and its mode of action is to generally function as an inhibitor for the enzyme.

The binding agent usually is a known ligand used as a control and is capable of binding to the natural wild-type enzyme and the mutant enzyme employed in the assay and is usually chosen as a known peptide inhibitor for the enzyme.

The binding agent also contains a known signal-producing agent to cause or induce the signal in the assay and can be an agent inducing e.g., phosphorescence or fluorescence (ELISA), color reaction or a scintillation signal.

In the instant embodiment, where the assay is a scintillation assay, the signal agent is a radionuclide, i.e., tritium, I^{125}, which induces the scintillant in the solid support to emit measurable light radiation, i.e., photon emission, which can be measured by using conventional scintillation and beta radiation counters.

We have also discovered that introducing two or more 4-phosphonodifluoromethyl phenylalanine (F_2Pmp) groups into a known binding agent greatly enhances the binding affinity of the

binding agent to the enzyme and improves its stability by rendering the resulting complex less susceptible to hydrolytic cleavage.

A method for introducing one F_2Pmp moiety into a ligand is known in the art and is described in detail in *Biochem. Biophys. Res. Comm.* Vol. 204, pp. 129-134 (1994) hereby incorporated by reference for this particular purpose.

As a result of this technology we discovered a new class of ligands having extremely good binding affinity for PTP1B. These include:

N-Benzoyl-L-glutamyl-[4-phosphono(difluoromethyl)]-L-phenyl-alanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide,
N-Acetyl-L-glutamyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide,
L-Glutamyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide,
L-Lysinyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide,
L-Serinyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide,
L-Prolinyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide, and
L-Isoleucinyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide.

A useful ligand in the series is Bz-NEJJ-$CONH_2$, whose chemical name is: N-Benzoyl-L-glutamyl-[4-phosphono(difluoro-methyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenyl-alanineamide, and its tritiated form, N-(3,5-Ditritio)benzoyl-L-glutamyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono-(dilfuoromethyl)]-L-phenylalanineamide.

Synthesis of both cold and hot ligands is described in the Examples.

The following Examples are illustrative of carrying out the invention and should not be construed as being limitations on the scope or spirit of the instant invention.

EXAMPLES

1. Preparation of PTP1B Truncate (Amino Acid Sequence from 1-320 and Fused GST-PTP1B Construct

An *E. coli* culture carrying a PET plasmid expressing the full length PTP1B protein was disclosed in J. Chernoff *et al. Proc Natl. Acad. Sci. USA* , 87, pp. 2735-2739, (1990). This was modified to a truncated PTP1B enzyme complex containing the active site with amino acids 1-320 inclusive, by the following procedure:

The full length human PTP-1B cDNA sequence (published in J. Chernoff et al., PNAS, USA, *supra*) cloned into a PET vector was obtained from Dr. Raymond Erickson (Harvard University). The PTP-1B cDNA sequence encoding amino acids 1-320 (Seq. ID No. 1) was amplified by PCR using the full length sequence as template. The 5' primer used for the amplification included a Bam HI site at the 5' end and the 3' primer had an Eco RI site at the 3' end. The amplified fragment was cloned into pCR2 (Invitrogen) and sequenced to insure that no sequence errors had been introduced by Taq polymerase during the amplification. This sequence was released from pCR2 by a Bam HI/Eco RI digest and the PTP-1B cDNA fragment ligated into the GST fusion vector pGEX-2T (Pharmacia) that had been digested with the same enzymes. The GST-PTP-1B fusion protein expressed in *E. Coli* has an active protein tyrosine phosphatase activity. This same 1-320 PTP-1B sequence (Seq. ID No. 1) was then cloned into the expression vector pFLAG-2, where FLAG is the octa-peptide AspTyrLysAspAspAspAspLys. This was done by releasing the PTP-1B sequence from the pGEX-2T vector by Nco I/Eco RI digest, filling in the ends of this fragment by Klenow and blunt-end ligating into the blunted Eco RI site of pFLAG2. Site-directed mutagenesis was performed on pFLAG2-PTP-1B plasmid using the Chameleon (Stratagene) double-stranded mutagenesis kit from Stratagene, to replaced the active-site Cys-215 with serine. The mutagenesis was carried out essentially as described by the manufacturer and mutants identifed by DNA sequencing. The FLAG-PTP-1B Cys215Ser mutant (Seq. ID No. 7) was expressed, purified and found not to have any phosphatase activity. The GST-

PTP-1B $Cys^{215}Ser$ mutant was made using the mutated $Cys^{215}Ser$ sequence of PTP-1B already cloned into pFLAG2, as follows. The pFLAG2- PTP-1B $Cys^{215}Ser$ plasmid (Seq. ID No. 7) was digested with Sal I (3' end of PTP-1B sequence), filled in using Klenow polymerase (New England Biolabs), the enzymes were heat inactivated and the DNA redigested with Bgl II. The 500 bp 3' PTP-1B cDNA fragment which is released and contains the mutated active site was recovered. The pGEX-2T-PTP-1B plasmid was digested with Eco RI (3' end of PTP-1B sequence), filled in by Klenow, phenol/chloroform extracted and ethanol precipitated. This DNA was then digested with Bgl II, producing two DNA fragments a 500 bp 3' PTP-1B cDNA fragment that contains the active site and a 5.5 Kb fragment containing the pGEX-2T vector plus the 5' end of PTP-1B. The 5.5 Kb pGEX-2T 5' PTP-1B fragment was recovered and ligated with the 500 bp Bgl II/Sal I fragment containing the mutated active site. The ligation was transformed into bacteria (type DH5α, G) and clones containing the mutated active site sequence identified by sequencing. The GST-PTP-1B $Cys^{215}Ser$ mutant was overexpressed, purified and found not to have any phosphatase activity.

2. Preparation of Tritiated Bz-NEJJ-$CONH_2$

This compound can be prepared as outlined in Scheme 1, below, and by following the procedures:

Synthesis of N-Benzoyl-L-glutamyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanineamide (BzN-EJJ-$CONH_2$)

1.0 g of TentaGel® S RAM resin (RAPP polymer, ~ 0.2 mmol/g) as represented by the shaded bead in Scheme 1, was treated with piperidine (3 mL) in DMF (5 mL) for 30 min. The resin (symbolized by the circular P, containing the remainder of the organic molecule except the amino group) was washed successively with DMF (3 x 10 mL) and CH_2Cl_2 (10 mL) and air dried. A solution of DMF (5 mL), N^{∞}-Fmoc-4-[diethylphosphono-(difluoromethyl)]-L-

phenylalanine (350 mg) , where Fmoc is 9-fluorenylmethoxycarbonyl, and O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluranium hexafluorphosphate,(acronym being HATU, 228 mg) was treated with diisopropyl-ethylamine (0.21 mL) and, after 15 min., was added to the resin in 3 mL of DMF. After 1 h, the resin was washed successively with DMF (3x10 mL) and CH_2Cl_2 (10 mL) and air dried. The sequence was repeated two times, first using N^{∞}-Fmoc-4-[diethylphosphono-(difluoromethyl)]-L-phenylalamine and then using N-Fmoc-L-glutamic acid gamma-*t*-butyl ester. After the final coupling, the resin bound tripeptide was treated with a mixture of piperidine (3 mL) in DMF (5mL) for 30 min. and was then washed successively with DMF (3x10 mL) and CH_2Cl_2 (10 mL) and air dried.

To a solution of benzoic acid (61 mg) and HATU (190 mg) in DMF (1 mL) was added diisopropylethylamine (0.17 mL) and, after 15 min. the mixture was added to a portion of the resin prepared above (290 mg) in 1 mL DMF. After 90 min. the resin was washed successively with DMF (3 x 10 mL) and CH_2Cl_2 (10 mL) and air dried. The resin was treated with 2 mL of a mixture of TFA: water (9:1) and 0.05 mL of triisopropylsilane (TIPS-H) for 1 h. The resin was filtered off and the filtrate was diluted with water (2 mL) and concentrated *in vacuo* at 35°C. The residue was treated with 2.5 mL of a mixture of TFA:DMS:TMSOTf (5:3:1) and 0.05 mL of TIPS-H, and stirred at 25°C for 15 h. (TFA is trifluoroacetic acid, DMS is dimethyl sulfate, TMSOTf is trimethylsilyl trifluoromethanesulfonate).

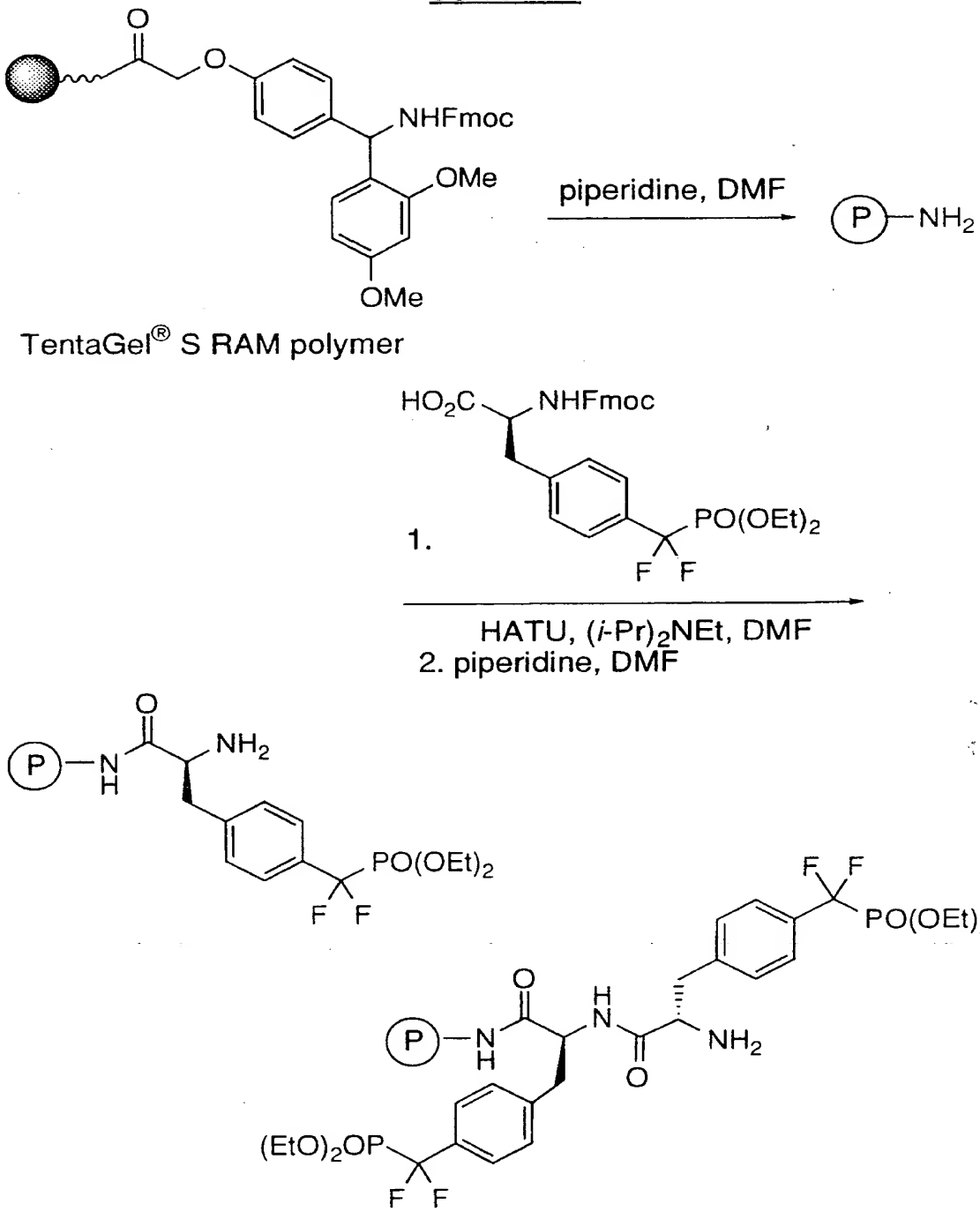
The desired tripeptide, the title compound, was purified by reverse phase HPLC (C18 column, 25 x 100 mm) using a mobile phase gradient from 0.2% TFA in water to 50/50 acetonitrile/0.2% TFA in water over 40 min. and monitoring at 230 nm. The fraction eluting at approximately 14.3 min. was collected, concentrated and lyophylized to yield the title compound as a white foam.

Synthesis of N-(3,5-Ditritio)benzoyl-L-glutamyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(dilfuoromethyl)]-L-phenylalanineamide

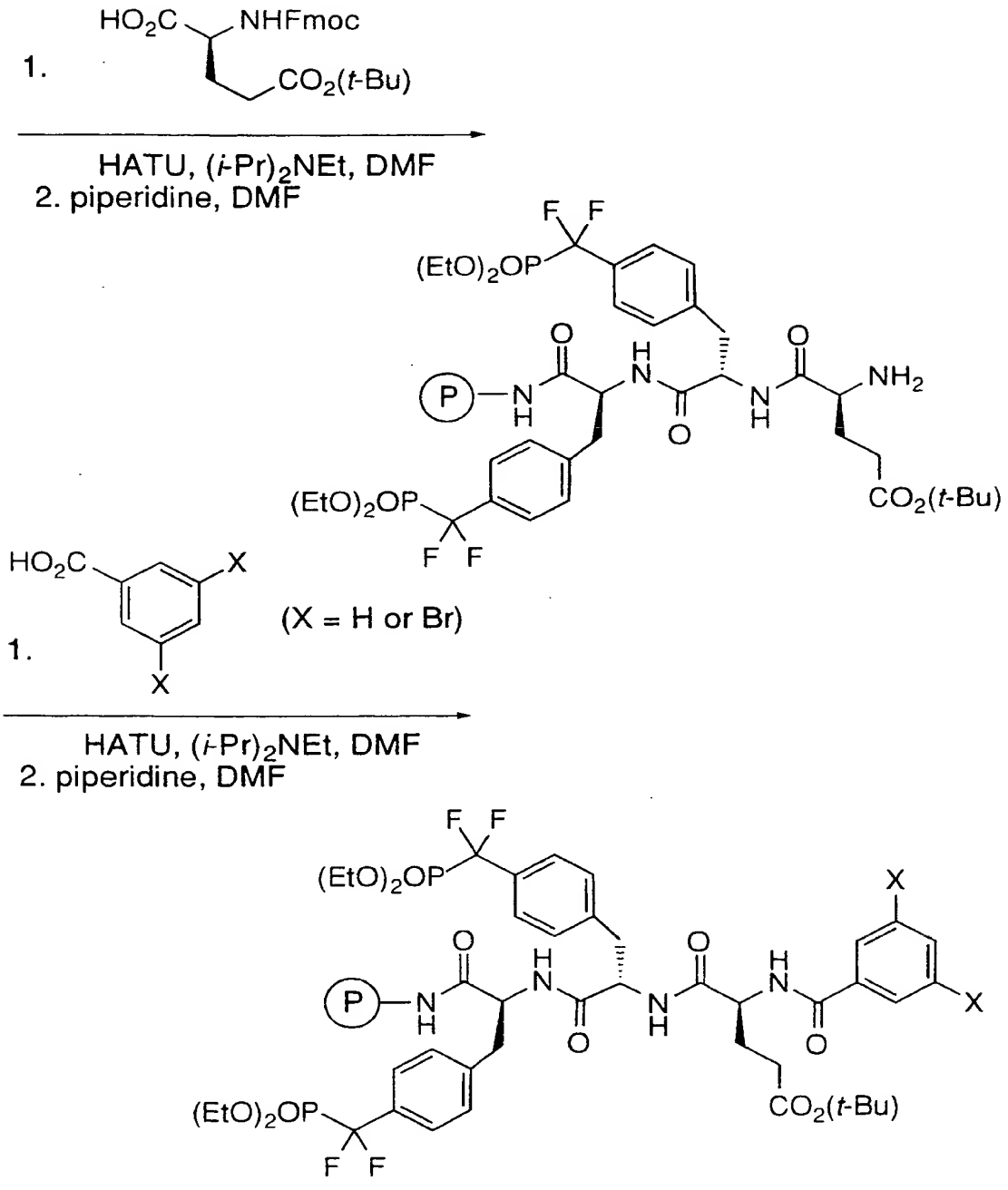
The above procedure described for the preparation of BzN-EJJ-$CONH_2$ was repeated, but substituting 3,5-dibromobenzoic acid for benzoic acid. After HPLC purification as before, except using a gradient over 30 min. and collecting the fraction at approximately 18.3 min., the dibromo containing tripeptide was obtained as a white foam.

A portion of this material (2 mg) was dissolved in methanol/triethylamine (0.5 mL, 4/1), 10% Pd-C (2 mg) was added, and the mixture stirred under an atmosphere of tritium gas for 24 h. The mixture was filtered through celite, washing with methanol and the filtrate was concentrated. The title compound was obtained after purification by semi-preparative HPLC using a C18 column and an isocratic mobile phase of acetonitrile/0.2% TFA in water (15:100). The fraction eluting at approximately 5 min. was collected and concentrated *in vacuo*. The title compound was dissolved in 10 mL of methanol/water (9:1) to provide a 0.1 mg/mL solution of specific activity 39.4 Ci/mmol.

SCHEME 1



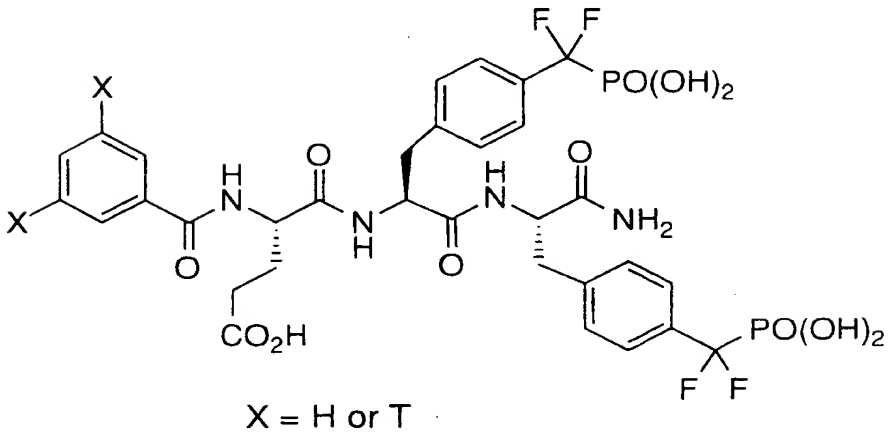
SCHEME 1 CONT'D



SCHEME 1 CONT'D

1. TFA-H_2O (9:1)
2. TFA-DMS-TMSOTf-TIPSH
3. HPLC purification

4. for X = Br: T_2 (g), 10% Pd-C
   MeOH, Et_3N;
   HPLC purification



X = H or T

By following the above described procedure for BzN-EJJ-$CONH_2$, the following other peptide inhibitors were also similarly prepared:

N-Benzoyl-L-glutamyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide,
N-Acetyl-L-glutamyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide,
L-Glutamyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide,
L-Lysinyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide,
L-Serinyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide,
L-Prolinyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide, and

L-Isoleucinyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide.

4. Phosphatase Assay Protocol

Materials:

EDTA - ethylenediaminetetraacetic acid (Sigma)

DMH - N,N'-dimethyl-N,N'-bis(mercaptoacetyl)-hydrazine (synthesis published in *J. Org. Chem.* 56, pp. 2332-2337,(1991) by R. Singh and G.M. Whitesides and can be substituted with DTT - dithiothreitol Bistris - 2,2-bis(hydroxymethyl)2,2',2"-nitrilotriethanol-(Sigma) Triton X-100 - octylphenolpoly(ethylene-glycolether) 10 (Pierce)

Antibody: Anti-glutathione S-transferase rabbit (H and L) fraction (Molecular Probes)

Enzyme: Human recombinant PTP1B, containing amino acids 1-320, (Seq. ID No. 1) fused to GST enzyme (glutathione S-transferase) purified by affinity chromatography. Wild type (Seq. ID No. 1) contains active site cysteine(215), whereas mutant (Seq. ID No. 7) contains active site serine(215).

Tritiated peptide: Bz-NEJJ-$CONH_2$, Mwt. 808, empirical formula, $C_{32}H_{32}T_2O_{12}P_2F_4$

Stock Solutions

(10X) Assay Buffer	500 mM Bistris (Sigma), pH 6.2, MW=209.2 20mM EDTA (GIBCO/BRL) Store at 4° C.

Prepare fresh daily:

Assay Buffer (1X) (room temp.)	50 mM Bistris 2 mM EDTA 5 mM DMH (MW=208)

Enzyme Dilution

Buffer (keep on ice)	50 mM Bistris
	2 mM EDTA
	5 mM DMH
	20% Glycerol (Sigma)
	0.01 mg/ml Triton X-100 (Pierce)

Antibody Dilution

Buffer (keep on ice)	50 mM Bistris
	2 mM EDTA

IC_{50} Binding Assay Protocol:

Compounds (ligands) which potentially inhibit the binding of a radioactive ligand to the specific phosphatase are screened in a 96-well plate format as follows:

To each well is added the following solutions @ 25°C in the following chronological order:

1. 110 µl of assay buffer.
2. 10 µl. of 50 nM tritiated BzN-EJJ-$CONH_2$ in assay buffer (1X) @ 25°C.
3. 10 µl. of testing compound in DMSO at 10 different concentrations in serial dilution (final DMSO, about 5% v/v) in duplicate @ 25°C.
4. 10 µl. of 3.75 µg/ml purified human recombinant GST-PTP1B in enzyme dilution buffer.
5. The plate is shaken for 2 minutes.
6. 10 µl. of 0.3 µg/ml anti-glutathione S-transferase (anti-GST) rabbit IgG (Molecular Probes) diluted in antibody dilution buffer @ 25°C.
7. The plate is shaken for 2 minutes.
8. 50 µl. of protein A-PVT SPA beads (Amersham) @ 25°C.
9. The plate is shaken for 5 minutes. The binding signal is quantified on a Microbeta 96-well plate counter.
10. The non-specific signal is defined as the enzyme-ligand binding in the absence of anti-GST antibody.

11. 100% binding activity is defined as the enzyme-ligand binding in the presence of anti-GST antibody, but in the absence of the testing ligands with the non-specific binding subtracted.

12. Percentage of inhibition is calculated accordingly.

13. IC_{50} value is approximated from the non-linear regression fit with the 4-parameter/multiple sites equation (described in: "Robust Statistics", New York, Wiley, by P.J. Huber (1981) and reported in nM units.

14. Test ligands (compounds) with larger than 90% inhibition at 10 μM are defined as actives.

The following Table I illustrates typical assay results of examples of known compounds which competitively inhibit the binding of the binding agent, BzN-EJJ-CONH2.

TABLE I

GST-PTP1B SPA Binding Assay with Non-Mutated (Cys215) and Mutated enzyme (Ser215)

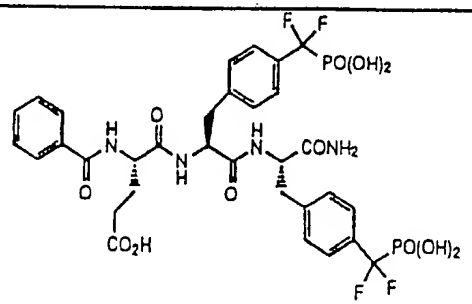
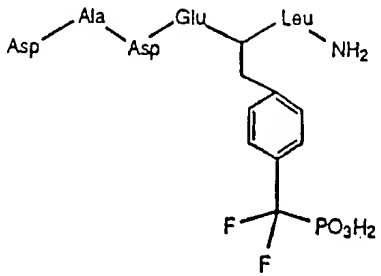
Compound	Structure	Non-Mutated	Mutated
Control:			
Tripeptide(F2PMP)2		14 nM	8 nM
DADE(F2PMP)L hexapeptide (T. Burke et al, Biochem. Biophys. Res. Comm. 204, 129, (1994))		400 nM	100 nM

TABLE I (Cont'd.)

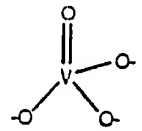
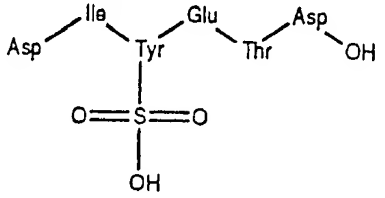
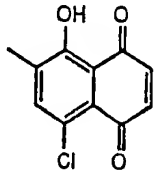
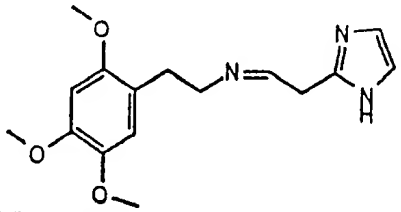
SH-specific binding:			
Vanadate		2 µM	>100 µM
Insulin Receptor Peptide		17 µM	70 µM
Potential Oxidizing agents:			
Hydrogen peroxide	H_2O_2	90% at 83 µM	0% at 83 µM
Quinone		4 µM	>100 µM
Potential Alkylating agents:			
Imine		67% at 2 µM	10% at 2 µM

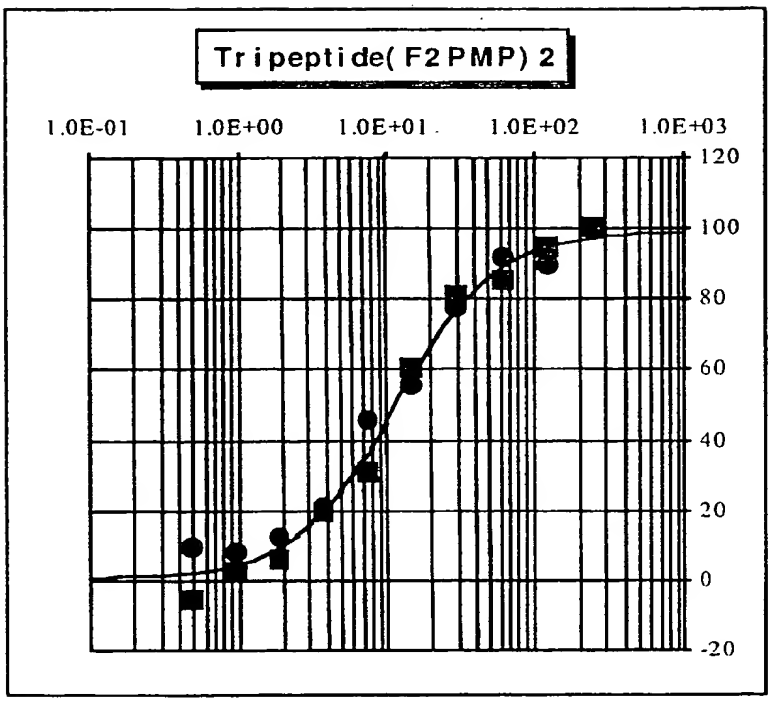
TABLE II

Raw Data, Counts (dpm)
(duplicates)

	no antibody (- control)	antibody (+ control)	250	125	conc. BzN-EJJ-CONH2, nM 62.5	31.25	15.625	7.813	3.906	1.953	0.977	0.488
dpm	252	5652	288	873	757	1550	2775	3367	4743	5220	5454	5384
dpm	304	6380	273	588	1109	1337	2525	4165	4838	5581	5781	6211

TABLE III

	no antibody (- control)	antibody (+ control)	250	125	conc. Bz-EJJ-CONH2, nM 62.5	31.25	15.625	7.813	3.906	1.953	0.977	0.488
% Inh	100	5	100	90	92	78	56	45	21	12	8	9
% Inh	100	-8	100	95	85	81	60	30	19	6	2	-5



Preparation of Cathepsin K(O2) Mutant (CAT-K Mutant)

Cathepsin K is a prominent cysteine protease in human osteoclasts and is believed to play a key role in osteoclast-mediated bone resorption. Inhibitors of cathepsin K will be useful for the treatment of bone disorders (such as osteoporosis) where excessive bone resorption occurs. Cathepsin K is synthesized as a dormant preproenzyme (Seq. ID No. 4). Both the pre-domain (Met^{1}-Ala^{15}) and the prodomain (Leu^{16}-Arg^{114}) must be removed for full catalytic activity. The mature form of the protease (Ala^{115}-Met^{329}) contains the active site Cys residue (Cys^{139}).

The mature form of cathepsin K is engineered for expression in bacteria and other recombinant systems as a Met Ala^{115}-Met^{329} construct by PCR-directed template modification of a clone that is identified. Epitope-tagged variants are also generated: (Met[FLAG]Ala^{115}-Met^{329} and Met Ala^{115}-Met^{329}[FLAG]; where FLAG is the octa-peptide AspTyrLysAspAspAspAspLys). For the purpose of establishing a binding assay, several other constructs are generated including Met[FLAG]Ala^{115}-[Cys^{139} to Ser^{139}]-Met^{329} and Met Ala^{115}-[Cys^{139} to Ser^{139}]-Met^{329}[FLAG] (where the active site Cys is mutated to a Ser residue), and Met[FLAG]Ala^{115}-[Cys^{139} to Ala^{139}]-Met^{329} and Met Ala^{115}-[Cys^{139} to Ala^{139}]-Met^{329}[FLAG] (where the active site Cys is mutated to an Ala residue). In all cases, the resulting re-engineered polypeptides can be used in a binding assay by tethering the mutated enzymes to SPA beads via specific anti-FLAG antibodies that are commercially available (IDI-KODAK). Other epitope tags, GST and other fusions can also be used for this purpose and binding assay formats other than SPA can also be used. Ligands based on the prefered substrate for cathepsin K (e.g. Ac-P_2-P_1, Ac-P_2-P_1-aldehydes, Ac-P_2-P_1-ketones; where P1 is an amino acid with a hydrophilic side chain, preferably Arg or Lys, and P2 is an amino acid with a small hydrophobic side chain, preferably Leu, Val or Phe) are suitable in their radiolabeled (tritiated) forms for SPA-based binding assays. Similar binding assays can also be established for other cathepsin family members.

Preparation of Apopain (capsase-3) Mutant

Apopain is the active form of a cysteine protease belonging to the capsase superfamily of ICE/CED-3 like enzymes. It is derived from a catalytically dormant proenzyme that contains both the 17 kDa large subunit (p17) and 12 kDa (p12) small subunit of the catalytically active enzyme within a 32 kDa proenzyme polypeptide (p32). Apopain is a key mediator in the effector mechanism of apoptotic cell death and modulators of the activity of this enzyme, or structurally-related isoforms, will be useful for the therapeutic treatment of diseases where inappropriate apoptosis is prominent, e.g., Alzheimer's disease.

The method used for production of apopain involves folding of active enzyme from its constituent p17 and p12 subunits which are expressed separately in *E. coli*. The apopain p17 subunit (Ser^{29}-Asp^{175}) and p12 subunit (Ser^{176}-His^{277}) are engineered for expression as $MetSer^{29}$-Asp^{175} and $MetSer^{176}$-His^{277} constructs, respectively, by PCR-directed template modification. For the purpose of establishing a binding assay, several other constructs are generated, including a $MetSer^{29}$-[Cys^{163} to Ser^{163}]-Asp^{175} large subunit and a Met^{1}-[Cys^{163} to Ser^{163}]-His^{277} proenzyme. In the former case, the active site Cys residue in the large subunit (p17) is replaced with a Ser residue by site-directed mutagenesis. This large subunit is then re-folded with the recombinant p12 subunit to generate the mature form of the enzyme except with the active site Cys mutated to a Ser. In the latter case, the same Cys^{163} to Ser^{163} mutation is made, except that the entire proenzyme is expressed. In both cases, the resulting re-engineered polypeptides can be used in a binding assay by tethering the mutated enzymes to SPA beads via specific antibodies that are generated to recognize apopain (antibodies against the prodomain, the large p17 subunit, the small p12 subunit and the entire p17:p12 active enzyme have been generated). Epitope tags or GST and other fusions could also be used for this purpose and binding assay formats other than SPA can also be used.

Ligands based on the prefered substrate for apopain (varients of AspGluValAsp), such as Ac- AspGluValAsp, Ac-AspGluValAsp-aldehydes, Ac-AspGluValAsp-ketones are suitable in their radiolabeled forms for SPA-based binding assays. Similar binding assays can also be established for other capsase family members.

DESCRIPTION OF THE SEQUENCE LISTINGS

SEQ ID NO. 1 is the top sense DNA strand of Figures 2A and 2B for the PTP1B tyrosine phosphatase enzyme.

SEQ ID NO. 2 is the amino acid sequence of Figures 2A and 2B for the PTP1B tyrosine phosphatase enzyme.

SEQ ID NO. 3 is the top sense cDNA strand of Figures 3A, 3B and 3C for the Cathepsin K preproenzyme.

SEQ ID NO. 4 is the amino acid sequence of Figures 3A, 3B and 3C for the Cathepsin K preproenzyme.

SEQ ID NO. 5 is the top sense cDNA strand of Figures 4A and 4B for the CPP32 apopain proenzyme.

SEQ ID NO. 6 is the amino acid sequence of Figures 4A and 4B for the CPP32 apopain proenzyme.

SEQ ID NO. 7 is the cDNA sequence of the human PTP-$1B_{1-320}$ Ser mutant.

SEQ ID NO. 8 is the amino acid sequence of the human PTP-$1B_{1-320}$ Ser mutant.

SEQ ID NO. 9 is the cDNA sequence for apopain C163S mutant.

SEQ ID NO. 10 is the amino acid sequence for the apopain C163S mutant.

SEQ ID NO. 11 is the large subunit of the heterodimeric amino acid sequence for the apopain C163S mutant.

SEQ ID NO. 12 is the cDNA sequence for the Cathepsin K C139S mutant.

SEQ ID NO. 13 is the cDNA sequence for the Cathepsin K C139A mutant.

SEQ ID NO. 14 is the amino acid sequence for the Cathepsin K C139S mutant.

SEQ ID NO. 15 is the amino acid sequence for the Cathepsin K C139A mutant.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Desmarais, Sylvie
Friesen, Richard
Zamboni, Richard

(ii) TITLE OF INVENTION: NEW LIGANDS FOR PHOSPHATASE BINDING ASSAY

(iii) NUMBER OF SEQUENCES: 15

(iv) CORRESPONDENCE ADDRESS:
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(C) CITY: RAHWAY
(D) STATE: NEW JERSEY
(E) COUNTRY: USA
(F) ZIP: 07065

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: FastSEQ for Windows Version 2.0

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US unknown
(B) FILING DATE: 04-NOV-1996
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: North, Robert J.
(B) REGISTRATION NUMBER: 27,366
(C) REFERENCE/DOCKET NUMBER: 19840 PCT

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 732-594-7262
(B) TELEFAX: 732-594-4720

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 963 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CAAGAAGATA ATGACTATAT CAACGCTAGT TTGATAAAAA TGGAAGAAGC CCAAAGGAGT     240
TACATTCTTA CCCAGGGCCC TTTGCCTAAC ACATGCGGTC ACTTTTGGGA GATGGTGTGG     300
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ATCATGGGGG ACTCTTCCGT GCAGGATCAG TGGAAGGAGC TTTCCCACGA GGACCTGGAG     900
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 320 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Glu Met Glu Lys Glu Phe Glu Gln Ile Asp Lys Ser Gly Ser Trp
 1               5                  10                  15
Ala Ala Ile Tyr Gln Asp Ile Arg His Glu Ala Ser Asp Phe Pro Cys
            20                  25                  30
Arg Val Ala Lys Leu Pro Lys Asn Lys Asn Arg Asn Arg Tyr Arg Asp
        35                  40                  45
Val Ser Pro Phe Asp His Ser Arg Ile Lys Leu His Gln Glu Asp Asn
    50                  55                  60
Asp Tyr Ile Asn Ala Ser Leu Ile Lys Met Glu Glu Ala Gln Arg Ser
65                  70                  75                  80
Tyr Ile Leu Thr Gln Gly Pro Leu Pro Asn Thr Cys Gly His Phe Trp
                85                  90                  95
Glu Met Val Trp Glu Gln Lys Ser Arg Gly Val Val Met Leu Asn Arg
            100                 105                 110
```

```
Val Met Glu Lys Gly Ser Leu Lys Cys Ala Gln Tyr Trp Pro Gln Lys
        115                 120                 125
Glu Glu Lys Glu Met Ile Phe Glu Asp Thr Asn Leu Lys Leu Thr Leu
    130                 135                 140
Ile Ser Glu Asp Ile Lys Ser Tyr Tyr Thr Val Arg Gln Leu Glu Leu
145                 150                 155                 160
Glu Asn Leu Thr Thr Gln Glu Thr Arg Glu Ile Leu His Phe His Tyr
                165                 170                 175
Thr Thr Trp Pro Asp Phe Gly Val Pro Glu Ser Pro Ala Ser Phe Leu
            180                 185                 190
Asn Phe Leu Phe Lys Val Arg Glu Ser Gly Ser Leu Ser Pro Glu His
        195                 200                 205
Gly Pro Val Val Val His Cys Ser Ala Gly Ile Gly Arg Ser Gly Thr
    210                 215                 220
Phe Cys Leu Ala Asp Thr Cys Leu Leu Leu Met Asp Lys Arg Lys Asp
225                 230                 235                 240
Pro Ser Ser Val Asp Ile Lys Lys Val Leu Leu Glu Met Arg Lys Phe
                245                 250                 255
Arg Met Gly Leu Ile Gln Thr Ala Asp Gln Leu Arg Phe Ser Tyr Leu
            260                 265                 270
Ala Val Ile Glu Gly Ala Lys Phe Ile Met Gly Asp Ser Ser Val Gln
        275                 280                 285
Asp Gln Trp Lys Glu Leu Ser His Glu Asp Leu Glu Pro Pro Pro Glu
    290                 295                 300
His Ile Pro Pro Pro Pro Arg Pro Pro Lys Arg Ile Leu Glu Pro His
305                 310                 315                 320
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1669 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
GAAACAAGCA CTGGATTCCA TATCCCACTG CCAAAACCGC ATGGTTCAGA TTATCGCTAT     60
TGCAGCTTTC ATCATAATAC ACACCTTTGC TGCCGAAACG AAGCCAGACA ACAGATTTCC    120
ATCAGCAGGA TGTGGGGGCT CAAGGTTCTG CTGCTACCTG TGGTGAGCTT TGCTCTGTAC    180
CCTGAGGAGA TACTGGACAC CCACTGGGAG CTATGGAAGA AGACCCACAG GAAGCAATAT    240
AACAACAAGG TGGATGAAAT CTCTCGGCGT TTAATTTGGG AAAAAAACCT GAAGTATATT    300
TCCATCCATA ACCTTGAGGC TTCTCTTGGT GTCCATACAT ATGAACTGGC TATGAACCAC    360
CTGGGGGACA TGACCAGTGA AGAGGTGGTT CAGAAGATGA CTGGACTCAA AGTACCCCTG    420
TCTCATTCCC GCAGTAATGA CACCCTTTAT ATCCCAGAAT GGGAAGGTAG AGCCCCAGAC    480
TCTGTCGACT ATCGAAAGAA AGGATATGTT ACTCCTGTCA AAAATCAGGG TCAGTGTGGT    540
TCCTGTTGGG CTTTTAGCTC TGTGGGTGCC CTGGAGGGCC AACTCAAGAA GAAAACTGGC    600
AAACTCTTAA ATCTGAGTCC CCAGAACCTA GTGGATTGTG TGTCTGAGAA TGATGGCTGT    660
GGAGGGGGCT ACATGACCAA TGCCTTCCAA TATGTGCAGA AGAACCGGGG TATTGACTCT    720
```

GAAGATGCCT ACCCATATGT GGGACAGGAA GAGAGTTGTA TGTACAACCC AACAGGCAAG 780
GCAGCTAAAT GCAGAGGGTA CAGAGAGATC CCCGAGGGGA ATGAGAAAGC CCTGAAGAGG 840
GCAGTGGCCC GAGTGGGACC TGTCTCTGTG GCCATTGATG CAAGCCTGAC CTCCTTCCAG 900
TTTTACAGCA AAGGTGTGTA TTATGATGAA AGCTGCAATA GCGATAATCT GAACCATGCG 960
GTTTTGGCAG TGGGATATGG AATCCAGAAG GGAAACAAGC ACTGGATAAT TAAAAACAGC 1020
TGGGGAGAAA ACTGGGGAAA CAAAGGATAT ATCCTCATGG CTCGAAATAA GAACAACGCC 1080
TGTGGCATTG CCAACCTGGC CAGCTTCCCC AAGATGTGAC TCCAGCCAGC CAAATCCATC 1140
CTGCTCTTCC ATTTCTTCCA CGATGGTGCA GTGTAACGAT GCACTTTGGA AGGGAGTTGG 1200
TGTGCTATTT TTGAAGCAGA TGTGGTGATA CTGAGATTGT CTGTTCAGTT TCCCCATTTG 1260
TTTGTGCTTC AAATGATCCT TCCTACTTTG CTTCTCTCCA CCCATGACCT TTTTCACTGT 1320
GGCCATCAGG ACTTTCCCTG ACAGCTGTGT ACTCTTAGGC TAAGAGATGT GACTACAGCC 1380
TGCCCCTGAC TGTGTTGTCC CAGGGCTGAT GCTGTACAGG TACAGGCTGG AGATTTTCAC 1440
ATAGGTTAGA TTCTCATTCA CGGGACTAGT TAGCTTTAAG CACCCTAGAG GACTAGGGTA 1500
ATCTGACTTC TCACTTCCTA AGTTCCCTTC TATATCCTCA AGGTAGAAAT GTCTATGTTT 1560
TCTACTCCAA TTCATAAATC TATTCATAAG TCTTTGGTAC AAGTTTACAT GATAAAAAGA 1620
AATGTGATTT GTCTTCCCTT CTTTGCACTT TTGAAATAAA GTATTTATC 1669

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 329 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Met Trp Gly Leu Lys Val Leu Leu Leu Pro Val Val Ser Phe Ala Leu
 1               5                  10                  15
Tyr Pro Glu Glu Ile Leu Asp Thr His Trp Glu Leu Trp Lys Lys Thr
            20                  25                  30
His Arg Lys Gln Tyr Asn Asn Lys Val Asp Glu Ile Ser Arg Arg Leu
        35                  40                  45
Ile Trp Glu Lys Asn Leu Lys Tyr Ile Ser Ile His Asn Leu Glu Ala
    50                  55                  60
Ser Leu Gly Val His Thr Tyr Glu Leu Ala Met Asn His Leu Gly Asp
65                  70                  75                  80
Met Thr Ser Glu Glu Val Val Gln Lys Met Thr Gly Leu Lys Val Pro
                85                  90                  95
Leu Ser His Ser Arg Ser Asn Asp Thr Leu Tyr Ile Pro Glu Trp Glu
            100                 105                 110
Gly Arg Ala Pro Asp Ser Val Asp Tyr Arg Lys Lys Gly Tyr Val Thr
        115                 120                 125
Pro Val Lys Asn Gln Gly Gln Cys Gly Ser Cys Trp Ala Phe Ser Ser
    130                 135                 140
Val Gly Ala Leu Glu Gly Gln Leu Lys Lys Lys Thr Gly Lys Leu Leu
145                 150                 155                 160
```

```
Asn Leu Ser Pro Gln Asn Leu Val Asp Cys Val Ser Glu Asn Asp Gly
                165                 170                 175
Cys Gly Gly Gly Tyr Met Thr Asn Ala Phe Gln Tyr Val Gln Lys Asn
            180                 185                 190
Arg Gly Ile Asp Ser Glu Asp Ala Tyr Pro Tyr Val Gly Gln Glu Glu
        195                 200                 205
Ser Cys Met Tyr Asn Pro Thr Gly Lys Ala Ala Lys Cys Arg Gly Tyr
    210                 215                 220
Arg Glu Ile Pro Glu Gly Asn Glu Lys Ala Leu Lys Arg Ala Val Ala
225                 230                 235                 240
Arg Val Gly Pro Val Ser Val Ala Ile Asp Ala Ser Leu Thr Ser Phe
                245                 250                 255
Gln Phe Tyr Ser Lys Gly Val Tyr Tyr Asp Glu Ser Cys Asn Ser Asp
            260                 265                 270
Asn Leu Asn His Ala Val Leu Ala Val Gly Tyr Gly Ile Gln Lys Gly
        275                 280                 285
Asn Lys His Trp Ile Ile Lys Asn Ser Trp Gly Glu Asn Trp Gly Asn
    290                 295                 300
Lys Gly Tyr Ile Leu Met Ala Arg Asn Lys Asn Asn Ala Cys Gly Ile
305                 310                 315                 320
Ala Asn Leu Ala Ser Phe Pro Lys Met
                325
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1001 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
CTGCAGGAAT TCGGCACGAG GGGTGCTATT GTGAGGCGGT TGTAGAAGTT AATAAAGGTA     60
TCCATGGAGA ACACTGAAAA CTCAGTGGAT TCAAAATCCA TTAAAAATTT GGAACCAAAG    120
ATCATACATG GAAGCGAATC AATGGACTCT GGAATATCCC TGGACAACAG TTATAAAATG    180
GATTATCCTG AGATGGGTTT ATGTATAATA ATTAATAATA AGAATTTTCA TAAGAGCACT    240
GGAATGACAT CTCGGTCTGG TACAGATGTC GATGCAGCAA ACCTCAGGGA AACATTCAGA    300
AACTTGAAAT ATGAAGTCAG GAATAAAAAT GATCTTACAC GTGAAGAAAT TGTGGAATTG    360
ATGCGTGATG TTTCTAAAGA AGATCACAGC AAAAGGAGCA GTTTTGTTTG TGTGCTTCTG    420
AGCCATGGTG AAGAAGGAAT AATTTTTGGA ACAAATGGAC CTGTTGACCT GAAAAAAATA    480
ACAAACTTTT TCAGAGGGGA TCGTTGTAGA AGTCTAACTG GAAAACCCAA ACTTTTCATT    540
ATTCAGGCCT GCCGTGGTAC AGAACTGGAC TGTGGCATTG AGACAGACAG TGGTGTTGAT    600
GATGACATGG CGTGTCATAA AATACCAGTG GAGGCCGACT TCTTGTATGC ATACTCCACA    660
GCACCTGGTT ATTATTCTTG GCGAAATTCA AAGGATGGCT CCTGGTTCAT CCAGTCGCTT    720
TGTGCCATGC TGAAACAGTA TGCCGACAAG CTTGAATTTA TGCACATTCT TACCCGGGTT    780
AACCGAAAGG TGGCAACAGA ATTTGAGTCC TTTTCCTTTG ACGCTACTTT TCATGCAAAG    840
```

AAACAGATTC CATGTATTGT TTCCATGCTC ACAAAAGAAC TCTATTTTTA TCACTAAAGA 900

AATGGTTGGT TGGTGGTTTT TTTTAGTTTG TATGCCAAGT GAGAAGATGG TATATTTGGT 960

ACTGTATTTC CCTCTCATTT TGACCTACTC TCATGCTGCA G 1001

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 277 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Met Glu Asn Thr Glu Asn Ser Val Asp Ser Lys Ser Ile Lys Asn Leu
 1               5                  10                  15
Glu Pro Lys Ile Ile His Gly Ser Glu Ser Met Asp Ser Gly Ile Ser
            20                  25                  30
Leu Asp Asn Ser Tyr Lys Met Asp Tyr Pro Glu Met Gly Leu Cys Ile
        35                  40                  45
Ile Ile Asn Asn Lys Asn Phe His Lys Ser Thr Gly Met Thr Ser Arg
    50                  55                  60
Ser Gly Thr Asp Val Asp Ala Ala Asn Leu Arg Glu Thr Phe Arg Asn
65                  70                  75                  80
Leu Lys Tyr Glu Val Arg Asn Lys Asn Asp Leu Thr Arg Glu Glu Ile
                85                  90                  95
Val Glu Leu Met Arg Asp Val Ser Lys Glu Asp His Ser Lys Arg Ser
            100                 105                 110
Ser Phe Val Cys Val Leu Leu Ser His Gly Glu Glu Gly Ile Ile Phe
        115                 120                 125
Gly Thr Asn Gly Pro Val Asp Leu Lys Lys Ile Thr Asn Phe Phe Arg
    130                 135                 140
Gly Asp Arg Cys Arg Ser Leu Thr Gly Lys Pro Lys Leu Phe Ile Ile
145                 150                 155                 160
Gln Ala Cys Arg Gly Thr Glu Leu Asp Cys Gly Ile Glu Thr Asp Ser
                165                 170                 175
Gly Val Asp Asp Asp Met Ala Cys His Lys Ile Pro Val Glu Ala Asp
            180                 185                 190
Phe Leu Tyr Ala Tyr Ser Thr Ala Pro Gly Tyr Tyr Ser Trp Arg Asn
        195                 200                 205
Ser Lys Asp Gly Ser Trp Phe Ile Gln Ser Leu Cys Ala Met Leu Lys
    210                 215                 220
Gln Tyr Ala Asp Lys Leu Glu Phe Met His Ile Leu Thr Arg Val Asn
225                 230                 235                 240
Arg Lys Val Ala Thr Glu Phe Glu Ser Phe Ser Phe Asp Ala Thr Phe
                245                 250                 255
His Ala Lys Lys Gln Ile Pro Cys Ile Val Ser Met Leu Thr Lys Glu
            260                 265                 270
Leu Tyr Phe Tyr His
        275
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 963 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
ATGGAGATGG AAAAGGAGTT CGAGCAGATC GACAAGTCCG GGAGCTGGGC GGCCATTTAC     60
CAGGATATCC GACATGAAGC CAGTGACTTC CCATGTAGAG TGGCCAAGCT TCCTAAGAAC    120
AAAAACCGAA ATAGGTACAG AGACGTCAGT CCCTTTGACC ATAGTCGGAT TAAACTACAT    180
CAAGAAGATA ATGACTATAT CAACGCTAGT TTGATAAAAA TGGAAGAAGC CCAAAGGAGT    240
TACATTCTTA CCCAGGGCCC TTTGCCTAAC ACATGCGGTC ACTTTTGGGA GATGGTGTGG    300
GAGCAGAAAA GCAGGGGTGT CGTCATGCTC AACAGAGTGA TGGAGAAAGG TTCGTTAAAA    360
TGCGCACAAT ACTGGCCACA AAAAGAAGAA AAAGAGATGA TCTTTGAAGA CACAAATTTG    420
AAATTAACAT TGATCTCTGA AGATATCAAG TCATATTATA CAGTGCGACA GCTAGAATTG    480
GAAAACCTTA CAACCCAAGA AACTCGAGAG ATCTTACATT TCCACTATAC CACATGGCCT    540
GACTTTGGAG TCCCTGAATC ACCAGCCTCA TTCTTGAACT TTCTTTTCAA AGTCCGAGAG    600
TCAGGGTCAC TCAGCCCGGA GCACGGGCCC GTTGTGGTGC ACAGCAGTGC AGGCATCGGC    660
AGGTCTGGAA CCTTCTGTCT GGCTGATACC TGCCTCCTGC TGATGGACAA GAGGAAAGAC    720
CCTTCTTCCG TTGATATCAA GAAAGTGCTG TTAGAAATGA GGAAGTTTCG GATGGGGTTG    780
ATCCAGACAG CCGACCAGCT GCGCTTCTCC TACCTGGCTG TGATCGAAGG TGCCAAATTC    840
ATCATGGGGG ACTCTTCCGT GCAGGATCAG TGGAAGGAGC TTTCCCACGA GGACCTGGAG    900
CCCCCACCCG AGCATATCCC CCCACCTCCC CGGCCACCCA AACGAATCCT GGAGCCACAC    960
TGA                                                                  963
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 322 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
Met Glu Met Glu Lys Glu Phe Glu Gln Ile Asp Lys Ser Gly Ser Trp
 1               5                  10                  15
Ala Ala Ile Tyr Gln Asp Ile Arg His Glu Ala Ser Asp Phe Pro Cys
            20                  25                  30
Arg Val Ala Lys Leu Pro Lys Asn Lys Asn Arg Asn Arg Tyr Arg Asp
        35                  40                  45
```

```
Val Ser Pro Phe Asp His Ser Arg Ile Lys Leu His Gln Glu Asp Asn
    50                  55                  60
Asp Tyr Ile Asn Ala Ser Leu Ile Lys Met Glu Glu Ala Gln Arg Ser
65                  70                  75                  80
Tyr Ile Leu Thr Gln Gly Pro Leu Pro Asn Thr Cys Gly His Phe Trp
                85                  90                  95
Glu Met Val Trp Glu Gln Lys Ser Arg Gly Val Val Met Leu Asn Arg
            100                 105                 110
Val Met Glu Lys Gly Ser Leu Lys Cys Ala Gln Tyr Trp Pro Gln Lys
        115                 120                 125
Glu Glu Lys Glu Met Ile Phe Glu Asp Thr Asn Leu Lys Leu Thr Leu
    130                 135                 140
Ile Ser Glu Asp Ile Lys Ser Tyr Tyr Thr Val Arg Gln Leu Glu Leu
145                 150                 155                 160
Glu Asn Leu Thr Thr Gln Glu Thr Arg Glu Ile Leu His Phe His Tyr
                165                 170                 175
Thr Thr Trp Pro Asp Phe Gly Val Pro Glu Ser Pro Ala Ser Phe Leu
            180                 185                 190
Asn Phe Leu Phe Lys Val Arg Glu Ser Gly Ser Leu Ser Pro Glu His
        195                 200                 205
Gly Pro Val Val Val His Ser Ser Ala Gly Ile Gly Thr Cys Gly Arg
    210                 215                 220
Ser Gly Thr Phe Cys Leu Ala Asp Thr Cys Leu Leu Leu Met Asp Lys
225                 230                 235                 240
Arg Lys Asp Pro Ser Ser Val Asp Ile Lys Lys Val Leu Leu Glu Met
                245                 250                 255
Arg Lys Phe Arg Met Gly Leu Ile Gln Thr Ala Asp Gln Leu Arg Phe
            260                 265                 270
Ser Tyr Leu Ala Val Ile Glu Gly Ala Lys Phe Ile Met Gly Asp Ser
        275                 280                 285
Ser Val Gln Asp Gln Trp Lys Glu Leu Ser His Glu Asp Leu Glu Pro
    290                 295                 300
Pro Pro Glu His Ile Pro Pro Pro Pro Arg Pro Pro Lys Arg Ile Leu
305                 310                 315                 320
Glu Pro
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1001 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
CTGCAGGAAT TCGGCACGAG GGGTGCTATT GTGAGGCGGT TGTAGAAGTT AATAAAGGTA     60
TCCATGGAGA ACACTGAAAA CTCAGTGGAT TCAAAATCCA TTAAAAATTT GGAACCAAAG    120
ATCATACATG GAAGCGAATC AATGGACTCT GGAATATCCC TGGACAACAG TTATAAAATG    180
GATTATCCTG AGATGGGTTT ATGTATAATA ATTAATAATA AGAATTTTCA TAAGAGCACT    240
GGAATGACAT CTCGGTCTGG TACAGATGTC GATGCAGCAA ACCTCAGGGA AACATTCAGA    300
AACTTGAAAT ATGAAGTCAG GAATAAAAAT GATCTTACAC GTGAAGAAAT TGTGGAATTG    360
```

```
ATGCGTGATG TTTCTAAAGA AGATCACAGC AAAAGGAGCA GTTTTGTTTG TGTGCTTCTG     420
AGCCATGGTG AAGAAGGAAT AATTTTTGGA ACAAATGGAC CTGTTGACCT GAAAAAAATA     480
ACAAACTTTT TCAGAGGGGA TCGTTGTAGA AGTCTAACTG GAAAACCCAA ACTTTTCATT     540
ATTCAGGCCT CCCGTGGTAC AGAACTGGAC TGTGGCATTG AGACAGACAG TGGTGTTGAT     600
GATGACATGG CGTGTCATAA AATACCAGTG GAGGCCGACT TCTTGTATGC ATACTCCACA     660
GCACCTGGTT ATTATTCTTG GCGAAATTCA AAGGATGGCT CCTGGTTCAT CCAGTCGCTT     720
TGTGCCATGC TGAAACAGTA TGCCGACAAG CTTGAATTTA TGCACATTCT TACCCGGGTT     780
AACCGAAAGG TGGCAACAGA ATTTGAGTCC TTTTCCTTTG ACGCTACTTT TCATGCAAAG     840
AAACAGATTC CATGTATTGT TTCCATGCTC ACAAAAGAAC TCTATTTTTA TCACTAAAGA     900
AATGGTTGGT TGGTGGTTTT TTTTAGTTTG TATGCCAAGT GAGAAGATGG TATATTTGGT     960
ACTGTATTTC CCTCTCATTT TGACCTACTC TCATGCTGCA G                        1001
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 277 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Met Glu Asn Thr Glu Asn Ser Val Asp Ser Lys Ser Ile Lys Asn Leu
 1               5                  10                  15
Glu Pro Lys Ile Ile His Gly Ser Glu Ser Met Asp Ser Gly Ile Ser
            20                  25                  30
Leu Asp Asn Ser Tyr Lys Met Asp Tyr Pro Glu Met Gly Leu Cys Ile
        35                  40                  45
Ile Ile Asn Asn Lys Asn Phe His Lys Ser Thr Gly Met Thr Ser Arg
    50                  55                  60
Ser Gly Thr Asp Val Asp Ala Ala Asn Leu Arg Glu Thr Phe Arg Asn
65                  70                  75                  80
Leu Lys Tyr Glu Val Arg Asn Lys Asn Asp Leu Thr Arg Glu Glu Ile
                85                  90                  95
Val Glu Leu Met Arg Asp Val Ser Lys Glu Asp His Ser Lys Arg Ser
            100                 105                 110
Ser Phe Val Cys Val Leu Leu Ser His Gly Glu Glu Gly Ile Ile Phe
        115                 120                 125
Gly Thr Asn Gly Pro Val Asp Leu Lys Lys Ile Thr Asn Phe Phe Arg
    130                 135                 140
Gly Asp Arg Cys Arg Ser Leu Thr Gly Lys Pro Lys Leu Phe Ile Ile
145                 150                 155                 160
Gln Ala Ser Arg Gly Thr Glu Leu Asp Cys Gly Ile Glu Thr Asp Ser
                165                 170                 175
Gly Val Asp Asp Asp Met Ala Cys His Lys Ile Pro Val Glu Ala Asp
            180                 185                 190
Phe Leu Tyr Ala Tyr Ser Thr Ala Pro Gly Tyr Tyr Ser Trp Arg Asn
        195                 200                 205
Ser Lys Asp Gly Ser Trp Phe Ile Gln Ser Leu Cys Ala Met Leu Lys
    210                 215                 220
```

```
Gln Tyr Ala Asp Lys Leu Glu Phe Met His Ile Leu Thr Arg Val Asn
225                 230                 235                 240
Arg Lys Val Ala Thr Glu Phe Glu Ser Phe Ser Phe Asp Ala Thr Phe
                245                 250                 255
His Ala Lys Lys Gln Ile Pro Cys Ile Val Ser Met Leu Thr Lys Glu
            260                 265                 270
Leu Tyr Phe Tyr His
        275
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 277 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
Met Glu Asn Thr Glu Asn Ser Val Asp Ser Lys Ser Ile Lys Asn Leu
 1               5                  10                  15
Glu Pro Lys Ile Ile His Gly Ser Glu Ser Met Asp Ser Gly Ile Ser
            20                  25                  30
Leu Asp Asn Ser Tyr Lys Met Asp Tyr Pro Glu Met Gly Leu Cys Ile
        35                  40                  45
Ile Ile Asn Asn Lys Asn Phe His Lys Ser Thr Gly Met Thr Ser Arg
    50                  55                  60
Ser Gly Thr Asp Val Asp Ala Ala Asn Leu Arg Glu Thr Phe Arg Asn
65                  70                  75                  80
Leu Lys Tyr Glu Val Arg Asn Lys Asn Asp Leu Thr Arg Glu Glu Ile
                85                  90                  95
Val Glu Leu Met Arg Asp Val Ser Lys Glu Asp His Ser Lys Arg Ser
            100                 105                 110
Ser Phe Val Cys Val Leu Leu Ser His Gly Glu Glu Gly Ile Ile Phe
        115                 120                 125
Gly Thr Asn Gly Pro Val Asp Leu Lys Lys Ile Thr Asn Phe Phe Arg
    130                 135                 140
Gly Asp Arg Cys Arg Ser Leu Thr Gly Lys Pro Lys Leu Phe Ile Ile
145                 150                 155                 160
Gln Ala Ser Arg Gly Thr Glu Leu Asp Cys Gly Ile Glu Thr Asp Ser
                165                 170                 175
Gly Val Asp Asp Asp Met Ala Cys His Lys Ile Pro Val Glu Ala Asp
            180                 185                 190
Phe Leu Tyr Ala Tyr Ser Thr Ala Pro Gly Tyr Tyr Ser Trp Arg Asn
        195                 200                 205
Ser Lys Asp Gly Ser Trp Phe Ile Gln Ser Leu Cys Ala Met Leu Lys
    210                 215                 220
Gln Tyr Ala Asp Lys Leu Glu Phe Met His Ile Leu Thr Arg Val Asn
225                 230                 235                 240
Arg Lys Val Ala Thr Glu Phe Glu Ser Phe Ser Phe Asp Ala Thr Phe
                245                 250                 255
His Ala Lys Lys Gln Ile Pro Cys Ile Val Ser Met Leu Thr Lys Glu
            260                 265                 270
Leu Tyr Phe Tyr His
        275
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 990 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ATGTGGGGGC TCAAGGTTCT GCTGCTACCT GTGGTGAGCT TTGCTCTGTA CCCTGAGGAG 60
ATACTGGACA CCCACTGGGA GCTATGGAAG AAGACCCACA GGAAGCAATA TAACAACAAG 120
GTGGATGAAA TCTCTCGGCG TTTAATTTGG GAAAAAAACC TGAAGTATAT TTCCATCCAT 180
AACCTTGAGG CTTCTCTTGG TGTCCATACA TATGAACTGG CTATGAACCA CCTGGGGGAC 240
ATGACCAGTG AAGAGGTGGT TCAGAAGATG ACTGGACTCA AAGTACCCCT GTCTCATTCC 300
CGCAGTAATG ACACCCTTTA TATCCCAGAA TGGGAAGGTA GAGCCCCAGA CTCTGTCGAC 360
TATCGAAAGA AAGGATATGT TACTCCTGTC AAAAATCAGG GTCAGTGTGG TTCCTCTTGG 420
GCTTTTAGCT CTGTGGGTGC CCTGGAGGGC CAACTCAAGA AGAAAACTGG CAAACTCTTA 480
AATCTGAGTC CCCAGAACCT AGTGGATTGT GTGTCTGAGA ATGATGGCTG TGGAGGGGGC 540
TACATGACCA ATGCCTTCCA ATATGTGCAG AAGAACCGGG GTATTGACTC TGAAGATGCC 600
TACCCATATG TGGGACAGGA AGAGAGTTGT ATGTACAACC CAACAGGCAA GGCAGCTAAA 660
TGCAGAGGGT ACAGAGAGAT CCCCGAGGGG AATGAGAAAG CCCTGAAGAG GGCAGTGGCC 720
CGAGTGGGAC CTGTCTCTGT GGCCATTGAT GCAAGCCTGA CCTCCTTCCA GTTTTACAGC 780
AAAGGTGTGT ATTATGATGA AAGCTGCAAT AGCGATAATC TGAACCATGC GGTTTTGGCA 840
GTGGGATATG GAATCCAGAA GGGAAACAAG CACTGGATAA TTAAAAACAG CTGGGGAGAA 900
AACTGGGGAA ACAAAGGATA TATCCTCATG GCTCGAAATA AGAACAACGC CTGTGGCATT 960
GCCAACCTGG CCAGCTTCCC CAAGATGTGA 990

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 990 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

ATGTGGGGGC TCAAGGTTCT GCTGCTACCT GTGGTGAGCT TTGCTCTGTA CCCTGAGGAG 60
ATACTGGACA CCCACTGGGA GCTATGGAAG AAGACCCACA GGAAGCAATA TAACAACAAG 120
GTGGATGAAA TCTCTCGGCG TTTAATTTGG GAAAAAAACC TGAAGTATAT TTCCATCCAT 180

```
AACCTTGAGG CTTCTCTTGG TGTCCATACA TATGAACTGG CTATGAACCA CCTGGGGGAC    240
ATGACCAGTG AAGAGGTGGT TCAGAAGATG ACTGGACTCA AAGTACCCCT GTCTCATTCC    300
CGCAGTAATG ACACCCTTTA TATCCCAGAA TGGGAAGGTA GAGCCCCAGA CTCTGTCGAC    360
TATCGAAAGA AAGGATATGT TACTCCTGTC AAAAATCAGG GTCAGTGTGG TTCCGCTTGG    420
GCTTTTAGCT CTGTGGGTGC CCTGGAGGGC CAACTCAAGA AGAAAACTGG CAAACTCTTA    480
AATCTGAGTC CCCAGAACCT AGTGGATTGT GTGTCTGAGA ATGATGGCTG TGGAGGGGGC    540
TACATGACCA ATGCCTTCCA ATATGTGCAG AAGAACCGGG GTATTGACTC TGAAGATGCC    600
TACCCATATG TGGGACAGGA AGAGAGTTGT ATGTACAACC CAACAGGCAA GGCAGCTAAA    660
TGCAGAGGGT ACAGAGAGAT CCCCGAGGGG AATGAGAAAG CCCTGAAGAG GGCAGTGGCC    720
CGAGTGGGAC CTGTCTCTGT GGCCATTGAT GCAAGCCTGA CCTCCTTCCA GTTTTACAGC    780
AAAGGTGTGT ATTATGATGA AAGCTGCAAT AGCGATAATC TGAACCATGC GGTTTTGGCA    840
GTGGGATATG GAATCCAGAA GGGAAACAAG CACTGGATAA TTAAAAACAG CTGGGGAGAA    900
AACTGGGGAA ACAAAGGATA TATCCTCATG GCTCGAAATA AGAACAACGC CTGTGGCATT    960
GCCAACCTGG CCAGCTTCCC CAAGATGTGA                                     990
```

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 329 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
Met Trp Gly Leu Lys Val Leu Leu Leu Pro Val Val Ser Phe Ala Leu
 1               5                  10                  15
Tyr Pro Glu Glu Ile Leu Asp Thr His Trp Glu Leu Trp Lys Lys Thr
            20                  25                  30
His Arg Lys Gln Tyr Asn Asn Lys Val Asp Glu Ile Ser Arg Arg Leu
        35                  40                  45
Ile Trp Glu Lys Asn Leu Lys Tyr Ile Ser Ile His Asn Leu Glu Ala
    50                  55                  60
Ser Leu Gly Val His Thr Tyr Glu Leu Ala Met Asn His Leu Gly Asp
65                  70                  75                  80
Met Thr Ser Glu Glu Val Val Gln Lys Met Thr Gly Leu Lys Val Pro
                85                  90                  95
Leu Ser His Ser Arg Ser Asn Asp Thr Leu Tyr Ile Pro Glu Trp Glu
            100                 105                 110
Gly Arg Ala Pro Asp Ser Val Asp Tyr Arg Lys Lys Gly Tyr Val Thr
        115                 120                 125
Pro Val Lys Asn Gln Gly Gln Cys Gly Ser Ser Trp Ala Phe Ser Ser
    130                 135                 140
Val Gly Ala Leu Glu Gly Gln Leu Lys Lys Lys Thr Gly Lys Leu Leu
145                 150                 155                 160
Asn Leu Ser Pro Gln Asn Leu Val Asp Cys Val Ser Glu Asn Asp Gly
                165                 170                 175
```

```
Cys Gly Gly Gly Tyr Met Thr Asn Ala Phe Gln Tyr Val Gln Lys Asn
            180                 185                 190
Arg Gly Ile Asp Ser Glu Asp Ala Tyr Pro Tyr Val Gly Gln Glu Glu
        195                 200                 205
Ser Cys Met Tyr Asn Pro Thr Gly Lys Ala Ala Lys Cys Arg Gly Tyr
    210                 215                 220
Arg Glu Ile Pro Glu Gly Asn Glu Lys Ala Leu Lys Arg Ala Val Ala
225                 230                 235                 240
Arg Val Gly Pro Val Ser Val Ala Ile Asp Ala Ser Leu Thr Ser Phe
                245                 250                 255
Gln Phe Tyr Ser Lys Gly Val Tyr Tyr Asp Glu Ser Cys Asn Ser Asp
            260                 265                 270
Asn Leu Asn His Ala Val Leu Ala Val Gly Tyr Gly Ile Gln Lys Gly
        275                 280                 285
Asn Lys His Trp Ile Ile Lys Asn Ser Trp Gly Glu Asn Trp Gly Asn
    290                 295                 300
Lys Gly Tyr Ile Leu Met Ala Arg Asn Lys Asn Asn Ala Cys Gly Ile
305                 310                 315                 320
Ala Asn Leu Ala Ser Phe Pro Lys Met
                325
```

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 329 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
Met Trp Gly Leu Lys Val Leu Leu Leu Pro Val Val Ser Phe Ala Leu
 1               5                  10                  15
Tyr Pro Glu Glu Ile Leu Asp Thr His Trp Glu Leu Trp Lys Lys Thr
            20                  25                  30
His Arg Lys Gln Tyr Asn Asn Lys Val Asp Glu Ile Ser Arg Arg Leu
        35                  40                  45
Ile Trp Glu Lys Asn Leu Lys Tyr Ile Ser Ile His Asn Leu Glu Ala
    50                  55                  60
Ser Leu Gly Val His Thr Tyr Glu Leu Ala Met Asn His Leu Gly Asp
65                  70                  75                  80
Met Thr Ser Glu Glu Val Val Gln Lys Met Thr Gly Leu Lys Val Pro
                85                  90                  95
Leu Ser His Ser Arg Ser Asn Asp Thr Leu Tyr Ile Pro Glu Trp Glu
            100                 105                 110
Gly Arg Ala Pro Asp Ser Val Asp Tyr Arg Lys Lys Gly Tyr Val Thr
        115                 120                 125
Pro Val Lys Asn Gln Gly Gln Cys Gly Ser Ala Trp Ala Phe Ser Ser
    130                 135                 140
Val Gly Ala Leu Glu Gly Gln Leu Lys Lys Lys Thr Gly Lys Leu Leu
145                 150                 155                 160
Asn Leu Ser Pro Gln Asn Leu Val Asp Cys Val Ser Glu Asn Asp Gly
                165                 170                 175
Cys Gly Gly Gly Tyr Met Thr Asn Ala Phe Gln Tyr Val Gln Lys Asn
            180                 185                 190
Arg Gly Ile Asp Ser Glu Asp Ala Tyr Pro Tyr Val Gly Gln Glu Glu
        195                 200                 205
```

```
Ser Cys Met Tyr Asn Pro Thr Gly Lys Ala Ala Lys Cys Arg Gly Tyr
    210                 215                 220
Arg Glu Ile Pro Glu Gly Asn Glu Lys Ala Leu Lys Arg Ala Val Ala
225                 230                 235                 240
Arg Val Gly Pro Val Ser Val Ala Ile Asp Ala Ser Leu Thr Ser Phe
                245                 250                 255
Gln Phe Tyr Ser Lys Gly Val Tyr Tyr Asp Glu Ser Cys Asn Ser Asp
            260                 265                 270
Asn Leu Asn His Ala Val Leu Ala Val Gly Tyr Gly Ile Gln Lys Gly
        275                 280                 285
Asn Lys His Trp Ile Ile Lys Asn Ser Trp Gly Glu Asn Trp Gly Asn
    290                 295                 300
Lys Gly Tyr Ile Leu Met Ala Arg Asn Lys Asn Asn Ala Cys Gly Ile
305                 310                 315                 320
Ala Asn Leu Ala Ser Phe Pro Lys Met
                325
```

WHAT IS CLAIMED:

1. A peptide comprising a ligand having binding affinity for a tyrosine phosphatase or cysteine protease, wherein said ligand contains two or more 4-phosphono(difluoromethyl) phenylalanine groups.

2. The peptide of Claim 1 wherein said ligand has a greater binding affinity than the corresponding ligand only containing one of said 4-phosphono(difluoromethyl) phenylalanine groups.

3. A peptide selected from the group consisting of:
N-Benzoyl-L-glutamyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanineamide (BzN-EJJ-$CONH_2$), where E is glutamic acid and J is 4-phosphono(difluoro-methyl)]-L-phenylalanyl;
N-Benzoyl-L-glutamyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide;
N-Acetyl-L-glutamyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanine amide;
L-Glutamyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono--(difluoromethyl)]-L-phenylalanine amide;
L-Lysinyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono-(difluoromethyl)]-L-phenylalanine amide;
L-Serinyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono-(difluoromethyl)]-L-phenylalanine amide;
L-Prolinyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono-(difluoromethyl)]-L-phenylalanine amide; and
L-Isoleucinyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono-(difluoromethyl)]-L-phenylalanine amide.

4. The peptide of Claim 3 in tritiated or I^{125} iodinated form.

5. A tritiated peptide, N-(3,5-Ditritio)benzoyl-L-glutamyl-[4-phosphono(difluoromethyl)]-L-phenylalanyl-[4-phosphono(difluoromethyl)]-L-phenylalanineamide.

6. A process for increasing the binding affinity of a ligand for a tyrosine phosphatase or cysteine protease comprising introducing into the ligand two or more 4-phosphono(difluoromethyl) phenylalanine groups.

5

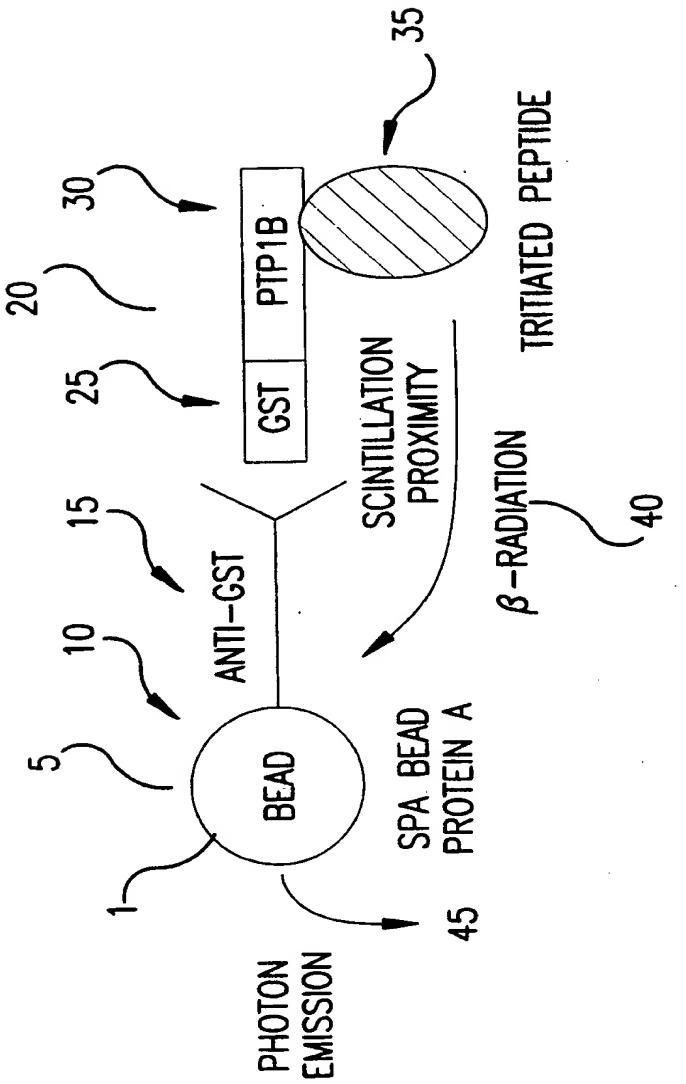

FIG.1

```
                ATGGAGATGGAAAAGGAGTTCGAGCAGATCGACAAGTCCGGGAGCTGGGCGGCCATTTAC
    1           ---------+---------+---------+---------+---------+---------+    60
                TACCTCTACCTTTTCCTCAAGCTCGTCTAGCTGTTCAGGCCCTCGACCCGCCGGTAAATG
    1           MetGluMetGluLysGluPheGluGlnIleAspLysSerGlySerTrpAlaAlaIleTyr    20

                CAGGATATCCGACATGAAGCCAGTGACTTCCCATGTAGAGTGGCCAAGCTTCCTAAGAAC
   61           ---------+---------+---------+---------+---------+---------+   120
                GTCCTATAGGCTGTACTTCGGTCACTGAAGGGTACATCTCACCGGTTCGAAGGATTCTTG
   21           GlnAspIleArgHisGluAlaSerAspPheProCysArgValAlaLysLeuProLysAsn    40

                AAAAACCGAAATAGGTACAGAGACGTCAGTCCCTTTGACCATAGTCGGATTAAACTACAT
  121           ---------+---------+---------+---------+---------+---------+   180
                TTTTTGGCTTTATCCATGTCTCTGCAGTCAGGGAAACTGGTATCAGCCTAATTTGATGTA
   41           LysAsnArgAsnArgTyrArgAspValSerProPheAspHisSerArgIleLysLeuHis    60

                CAAGAAGATAATGACTATATCAACGCTAGTTTGATAAAAATGGAAGAAGCCCAAAGGAGT
  181           ---------+---------+---------+---------+---------+---------+   240
                GTTCTTCTATTACTGATATAGTTGCGATCAAACTATTTTTACCTTCTTCGGGTTTCCTCA
   61           GlnGluAspAsnAspTyrIleAsnAlaSerLeuIleLysMetGluGluAlaGlnArgSer    80

                TACATTCTTACCCAGGGCCCTTTGCCTAACACATGCGGTCACTTTTGGGAGATGGTGTGG
  241           ---------+---------+---------+---------+---------+---------+   300
                ATGTAAGAATGGGTCCCGGGAAACGGATTGTGTACGCCAGTGAAAACCCTCTACCACACC
   81           TyrIleLeuThrGlnGlyProLeuProAsnThrCysGlyHisPheTrpGluMetValTrp   100

                GAGCAGAAAAGCAGGGGTGTCGTCATGCTCAACAGAGTGATGGAGAAAGGTTCGTTAAAA
  301           ---------+---------+---------+---------+---------+---------+   360
                CTCGTCTTTTCGTCCCCACAGCAGTACGAGTTGTCTCACTACCTCTTTCCAAGCAATTTT
  101           GluGlnLysSerArgGlyValValMetLeuAsnArgValMetGluLysGlySerLeuLys   120

                TGCGCACAATACTGGCCACAAAAAGAAGAAAAAGAGATGATCTTTGAAGACACAAATTTG
  361           ---------+---------+---------+---------+---------+---------+   420
                ACGCGTGTTATGACCGGTGTTTTTCTTCTTTTTCTCTACTAGAAACTTCTGTGTTTAAAC
  121           CysAlaGlnTyrTrpProGlnLysGluGluLysGluMetIlePheGluAspThrAsnLeu   140

                AAATTAACATTGATCTCTGAAGATATCAAGTCATATTATACAGTGCGACAGCTAGAATTG
  421           ---------+---------+---------+---------+---------+---------+   480
                TTTAATTGTAACTAGAGACTTCTATAGTTCAGTATAATATGTCACGCTGTCGATCTTAAC
  141           LysLeuThrLeuIleSerGluAspIleLysSerTyrTyrThrValArgGlnLeuGluLeu   160

                GAAAACCTTACAACCCAAGAAACTCGAGAGATCTTACATTTCCACTATACCACATGGCCT
  481           ---------+---------+---------+---------+---------+---------+   540
                CTTTTGGAATGTTGGGTTCTTTGAGCTCTCTAGAATGTAAAGGTGATATGGTGTACCGGA
  161           GluAsnLeuThrThrGlnGluThrArgGluIleLeuHisPheHisTyrThrThrTrpPro   180
```

FIG.2A

```
    GACTTTGGAGTCCCTGAATCACCAGCCTCATTCTTGAACTTTCTTTTCAAAGTCCGAGAG
541 ---------+---------+---------+---------+---------+---------+ 600
    CTGAAACCTCAGGGACTTAGTGGTCGGAGTAAGAACTTGAAAGAAAAGTTTCAGGCTCTC
181 AspPheGlyValProGluSerProAlaSerPheLeuAsnPheLeuPheLysValArgGlu 200

    TCAGGGTCACTCAGCCCGGAGCACGGGCCCGTTGTGGTGCACTGCAGTGCAGGCATCGGC
601 ---------+---------+---------+---------+---------+---------+ 660
    AGTCCCAGTGAGTCGGGCCTCGTGCCCGGGCAACACCACGTGACGTCACGTCCGTAGCCG
201 SerGlySerLeuSerProGluHisGlyProValValValHisCysSerAlaGlyIleGly 220

    AGGTCTGGAACCTTCTGTCTGGCTGATACCTGCCTCCTGCTGATGGACAAGAGGAAAGAC
661 ---------+---------+---------+---------+---------+---------+ 720
    TCCAGACCTTGGAAGACAGACCGACTATGGACGGAGGACGACTACCTGTTCTCCTTTCTG
221 ArgSerGlyThrPheCysLeuAlaAspThrCysLeuLeuLeuMetAspLysArgLysAsp 240

    CCTTCTTCCGTTGATATCAAGAAAGTGCTGTTAGAAATGAGGAAGTTTCGGATGGGGTTG
721 ---------+---------+---------+---------+---------+---------+ 780
    GGAAGAAGGCAACTATAGTTCTTTCACGACAATCTTTACTCCTTCAAAGCCTACCCCAAC
241 ProSerSerValAspIleLysLysValLeuLeuGluMetArgLysPheArgMetGlyLeu 260

    ATCCAGACAGCCGACCAGCTGCGCTTCTCCTACCTGGCTGTGATCGAAGGTGCCAAATTC
781 ---------+---------+---------+---------+---------+---------+ 840
    TAGGTCTGTCGGCTGGTCGACGCGAAGAGGATGGACCGACACTAGCTTCCACGGTTTAAG
261 IleGlnThrAlaAspGlnLeuArgPheSerTyrLeuAlaValIleGluGlyAlaLysPhe

    ATCATGGGGGACTCTTCCGTGCAGGATCAGTGGAAGGAGCTTTCCCACGAGGACCTGGAG
841 ---------+---------+---------+---------+---------+---------+ 900
    TAGTACCCCCTGAGAAGGCACGTCCTAGTCACCTTCCTCGAAAGGGTGCTCCTGGACCTC
    IleMetGlyAspSerSerValGlnAspGlnTrpLysGluLeuSerHisGluAspLeuGlu

    CCCCCACCCGAGCATATCCCCCCACCTCCCCGGCCACCCAAACGAATCCTGGAGCCACACTGA
901 ---------+---------+---------+---------+---------+---------+--- 960
    GGGGGTGGGCTCGTATAGGGGGGTGGAGGGGCCGGTGGGTTTGCTTAGGACCTCGGTGTGACT
301 ProProProGluHisIleProProProProArgProProLysArgIleLeuGluProHisEnd 320
```

FIG.2B

```
    GAAACAAGCACTGGATTCCATATCCCACTGCCAAAACCGCATGGTTCAGATTATCGCTAT
1   ---------+---------+---------+---------+---------+---------+   60
    CTTTGTTCGTGACCTAAGGTATAGGGTGACGGTTTTGGCGTACCAAGTCTAATAGCGATA

    TGCAGCTTTCATCATAATACACACCTTTGCTGCCGAAACGAAGCCAGACAACAGATTTCC
61  ---------+---------+---------+---------+---------+---------+  120
    ACGTCGAAAGTAGTATTATGTGTGGAAACGACGGCTTTGCTTCGGTCTGTTGTCTAAAGG

    ATCAGCAGGATGTGGGGGCTCAAGGTTCTGCTGCTACCTGTGGTGAGCTTTGCTCTGTAC
121 ---------+---------+---------+---------+---------+---------+  180
    TAGTCGTCCTACACCCCCGAGTTCCAAGACGACGATGGACACCACTCGAAACGAGACATG
              MetTrpGlyLeuLysValLeuLeuLeuProValValSerPheAlaLeuTyr

    CCTGAGGAGATACTGGACACCCACTGGGAGCTATGGAAGAAGACCCACAGGAAGCAATAT
181 ---------+---------+---------+---------+---------+---------+  240
    GGACTCCTCTATGACCTGTGGGTGACCCTCGATACCTTCTTCTGGGTGTCCTTCGTTATA
    ProGluGluIleLeuAspThrHisTrpGluLeuTrpLysLysThrHisArgLysGlnTyr

    AACAACAAGGTGGATGAAATCTCTCGGCGTTTAATTTGGGAAAAAAACCTGAAGTATATT
241 ---------+---------+---------+---------+---------+---------+  300
    TTGTTGTTCCACCTACTTTAGAGAGCCGCAAATTAAACCCTTTTTTTGGACTTCATATAA
    AsnAsnLysValAspGluIleSerArgArgLeuIleTrpGluLysAsnLeuLysTyrIle

    TCCATCCATAACCTTGAGGCTTCTCTTGGTGTCCATACATATGAACTGGCTATGAACCAC
301 ---------+---------+---------+---------+---------+---------+  360
    AGGTAGGTATTGGAACTCCGAAGAGAACCACAGGTATGTATACTTGACCGATACTTGGTG
    SerIleHisAsnLeuGluAlaSerLeuGlyValHisThrTyrGluLeuAlaMetAsnHis

    CTGGGGGACATGACCAGTGAAGAGGTGGTTCAGAAGATGACTGGACTCAAAGTACCCCTG
361 ---------+---------+---------+---------+---------+---------+  420
    GACCCCCTGTACTGGTCACTTCTCCACCAAGTCTTCTACTGACCTGAGTTTCATGGGGAC
    LeuGlyAspMetThrSerGluGluValValGlnLysMetThrGlyLeuLysValProLeu

    TCTCATTCCCGCAGTAATGACACCCTTTATATCCCAGAATGGGAAGGTAGAGCCCCAGAC
421 ---------+---------+---------+---------+---------+---------+  480
    AGAGTAAGGGCGTCATTACTGTGGGAAATATAGGGTCTTACCCTTCCATCTCGGGGTCTG
    SerHisSerArgSerAsnAspThrLeuTyrIleProGluTrpGluGlyArgAlaProAsp

    TCTGTCGACTATCGAAAGAAAGGATATGTTACTCCTGTCAAAAATCAGGGTCAGTGTGGT
481 ---------+---------+---------+---------+---------+---------+  540
    AGACAGCTGATAGCTTTCTTTCCTATACAATGAGGACAGTTTTTAGTCCCAGTCACACCA
    SerValAspTyrArgLysLysGlyTyrValThrProValLysAsnGlnGlyGlnCysGly
```

FIG.3A

```
    TCCTGTTGGGCTTTTAGCTCTGTGGGTGCCCTGGAGGGCCAACTCAAGAAGAAAACTGGC
541 ---------+---------+---------+---------+---------+---------+ 600
    AGGACAACCCGAAAATCGAGACACCCACGGGACCTCCCGGTTGAGTTCTTCTTTTGACCG
    SerCysTrpAlaPheSerSerValGlyAlaLeuGluGlyGlnLeuLysLysLysThrGly
       139

    AAACTCTTAAATCTGAGTCCCCAGAACCTAGTGGATTGTGTGTCTGAGAATGATGGCTGT
601 ---------+---------+---------+---------+---------+---------+ 660
    TTTGAGAATTTAGACTCAGGGGTCTTGGATCACCTAACACACAGACTCTTACTACCGACA
    LysLeuLeuAsnLeuSerProGlnAsnLeuValAspCysValSerGluAsnAspGlyCys

    GGAGGGGGCTACATGACCAATGCCTTCCAATATGTGCAGAAGAACCGGGGTATTGACTCT
661 ---------+---------+---------+---------+---------+---------+ 720
    CCTCCCCCGATGTACTGGTTACGGAAGGTTATACACGTCTTCTTGGCCCCATAACTGAGA
    GlyGlyGlyTyrMetThrAsnAlaPheGlnTyrValGlnLysAsnArgGlyIleAspSer

    GAAGATGCCTACCCATATGTGGGACAGGAAGAGAGTTGTATGTACAACCCAACAGGCAAG
721 ---------+---------+---------+---------+---------+---------+ 780
    CTTCTACGGATGGGTATACACCCTGTCCTTCTCTCAACATACATGTTGGGTTGTCCGTTC
    GluAspAlaTyrProTyrValGlyGlnGluGluSerCysMetTyrAsnProThrGlyLys

    GCAGCTAAATGCAGAGGGTACAGAGAGATCCCCGAGGGGAATGAGAAAGCCCTGAAGAGG
781 ---------+---------+---------+---------+---------+---------+ 840
    CGTCGATTTACGTCTCCCATGTCTCTCTAGGGGCTCCCCTTACTCTTTCGGGACTTCTCC
    AlaAlaLysCysArgGlyTyrArgGluIleProGluGlyAsnGluLysAlaLeuLysArg

    GCAGTGGCCCGAGTGGGACCTGTCTCTGTGGCCATTGATGCAAGCCTGACCTCCTTCCAG
841 ---------+---------+---------+---------+---------+---------+ 900
    CGTCACCGGGCTCACCCTGGACAGAGACACCGGTAACTACGTTCGGACTGGAGGAAGGTC
    AlaValAlaArgValGlyProValSerValAlaIleAspAlaSerLeuThrSerPheGln

    TTTTACAGCAAAGGTGTGTATTATGATGAAAGCTGCAATAGCGATAATCTGAACCATGCG
901 ---------+---------+---------+---------+---------+---------+ 960
    AAAATGTCGTTTCCACACATAATACTACTTTCGACGTTATCGCTATTAGACTTGGTACGC
    PheTyrSerLysGlyValTyrTyrAspGluSerCysAsnSerAspAsnLeuAsnHisAla

    GTTTTGGCAGTGGGATATGGAATCCAGAAGGGAAACAAGCACTGGATAATTAAAAACAGC
961 ---------+---------+---------+---------+---------+---------+ 1020
    CAAAACCGTCACCCTATACCTTAGGTCTTCCCTTTGTTCGTGACCTATTAATTTTTGTCG
    ValLeuAlaValGlyTyrGlyIleGlnLysGlyAsnLysHisTrpIleIleLysAsnSer

    TGGGGAGAAAACTGGGGAAACAAAGGATATATCCTCATGGCTCGAAATAAGAACAACGCC
1021---------+---------+---------+---------+---------+---------+ 1080
    ACCCCTCTTTTGACCCCTTTGTTTCCTATATAGGAGTACCGAGCTTTATTCTTGTTGCGG
    TrpGlyGluAsnTrpGlyAsnLysGlyTyrIleLeuMetAlaArgAsnLysAsnAsnAla
```

FIG.3B

```
      TGTGGCATTGCCAACCTGGCCAGCTTCCCCAAGATGTGACTCCAGCCAGCCAAATCCATC
1081  ---------+---------+---------+---------+---------+---------+  1140
      ACACCGTAACGGTTGGACCGGTCGAAGGGGTTCTACACTGAGGTCGGTCGGTTTAGGTAG
      CysGlyIleAlaAsnLeuAlaSerPheProLysMetEnd

      CTGCTCTTCCATTTCTTCCACGATGGTGCAGTGTAACGATGCACTTTGGAAGGGAGTTGG
1141  ---------+---------+---------+---------+---------+---------+  1200
      GACGAGAAGGTAAAGAAGGTGCTACCACGTCACATTGCTACGTGAAACCTTCCCTCAACC

      TGTGCTATTTTTGAAGCAGATGTGGTGATACTGAGATTGTCTGTTCAGTTTCCCCATTTG
1201  ---------+---------+---------+---------+---------+---------+  1260
      ACACGATAAAAACTTCGTCTACACCACTATGACTCTAACAGACAAGTCAAAGGGGTAAAC

      TTTGTGCTTCAAATGATCCTTCCTACTTTGCTTCTCTCCACCCATGACCTTTTTCACTGT
1261  ---------+---------+---------+---------+---------+---------+  1320
      AAACACGAAGTTTACTAGGAAGGATGAAACGAAGAGAGGTGGGTACTGGAAAAAGTGACA

      GGCCATCAGGACTTTCCCTGACAGCTGTGTACTCTTAGGCTAAGAGATGTGACTACAGCC
1321  ---------+---------+---------+---------+---------+---------+  1380
      CCGGTAGTCCTGAAAGGGACTGTCGACACATGAGAATCCGATTCTCTACACTGATGTCGG

      TGCCCCTGACTGTGTTGTCCCAGGGCTGATGCTGTACAGGTACAGGCTGGAGATTTTCAC
1381  ---------+---------+---------+---------+---------+---------+  1440
      ACGGGGACTGACACAACAGGGTCCCGACTACGACATGTCCATGTCCGACCTCTAAAAGTG

      ATAGGTTAGATTCTCATTCACGGGACTAGTTAGCTTTAAGCACCCTAGAGGACTAGGGTA
1441  ---------+---------+---------+---------+---------+---------+  1500
      TATCCAATCTAAGAGTAAGTGCCCTGATCAATCGAAATTCGTGGGATCTCCTGATCCCAT

      ATCTGACTTCTCACTTCCTAAGTTCCCTTCTATATCCTCAAGGTAGAAATGTCTATGTTT
1501  ---------+---------+---------+---------+---------+---------+  1560
      TAGACTGAAGAGTGAAGGATTCAAGGGAAGATATAGGAGTTCCATCTTTACAGATACAAA

      TCTACTCCAATTCATAAATCTATTCATAAGTCTTTGGTACAAGTTTACATGATAAAAAGA
1561  ---------+---------+---------+---------+---------+---------+  1620
      AGATGAGGTTAAGTATTTAGATAAGTATTCAGAAACCATGTTCAAATGTACTATTTTTCT

      AATGTGATTTGTCTTCCCTTCTTTGCACTTTTGAAATAAAGTATTTATC
1621  ---------+---------+---------+---------+---------  1669
      TTACACTAAACAGAAGGGAAGAAACGTGAAAACTTTATTTCATAAATAG
```

FIG.3C

```
                CTGCAGGAATTCGGCACGAGGGGTGCTATTGTGAGGCGGTTGTAGAAGTTAATAAAGGTA
1               ---------+---------+---------+---------+---------+---------+ 60
                GACGTCCTTAAGCCGTGCTCCCCACGATAACACTCCGCCAACATCTTCAATTATTTCCAT

                TCCATGGAGAACACTGAAAACTCAGTGGATTCAAAATCCATTAAAAATTTGGAACCAAAG
61              ---------+---------+---------+---------+---------+---------+ 120
                AGGTACCTCTTGTGACTTTTGAGTCACCTAAGTTTTAGGTAATTTTTAAACCTTGGTTTC
                   MetGluAsnThrGluAsnSerValAspSerLysSerIleLysAsnLeuGluProLys

                ATCATACATGGAAGCGAATCAATGGACTCTGGAATATCCCTGGACAACAGTTATAAAATG
121             ---------+---------+---------+---------+---------+---------+ 180
                TAGTATGTACCTTCGCTTAGTTACCTGAGACCTTATAGGGACCTGTTGTCAATATTTTAC
                IleIleHisGlySerGluSerMetAspSerGlyIleSerLeuAspAsnSerTyrLysMet

                GATTATCCTGAGATGGGTTTATGTATAATAATTAATAATAAGAATTTTCATAAGAGCACT
181             ---------+---------+---------+---------+---------+---------+ 240
                CTAATAGGACTCTACCCAAATACATATTATTAATTATTATTCTTAAAAGTATTCTCGTGA.
                AspTyrProGluMetGlyLeuCysIleIleIleAsnAsnLysAsnPheHisLysSerThr

                GGAATGACATCTCGGTCTGGTACAGATGTCGATGCAGCAAACCTCAGGGAAACATTCAGA
241             ---------+---------+---------+---------+---------+---------+ 300
                CCTTACTGTAGAGCCAGACCATGTCTACAGCTACGTCGTTTGGAGTCCCTTTGTAAGTCT
                GlyMetThrSerArgSerGlyThrAspValAspAlaAlaAsnLeuArgGluThrPheArg

                AACTTGAAATATGAAGTCAGGAATAAAAATGATCTTACACGTGAAGAAATTGTGGAATTG.
301             ---------+---------+---------+---------+---------+---------+ 360
                TTGAACTTTATACTTCAGTCCTTATTTTTACTAGAATGTGCACTTCTTTAACACCTTAAC
                AsnLeuLysTyrGluValArgAsnLysAsnAspLeuThrArgGluGluIleValGluLeu

                ATGCGTGATGTTTCTAAAGAAGATCACAGCAAAAGGAGCAGTTTTGTTTGTGTGCTTCTG
361             ---------+---------+---------+---------+---------+---------+ 420
                TACGCACTACAAAGATTTCTTCTAGTGTCGTTTTCCTCGTCAAAACAAACACACGAAGAC
                MetArgAspValSerLysGluAspHisSerLysArgSerSerPheValCysValLeuLeu

                AGCCATGGTGAAGAAGGAATAATTTTTGGAACAAATGGACCTGTTGACCTGAAAAAAATA
421             ---------+---------+---------+---------+---------+---------+ 480
                TCGGTACCACTTCTTCCTTATTAAAAACCTTGTTTACCTGGACAACTGGACTTTTTTTAT
                SerHisGlyGluGluGlyIleIlePheGlyThrAsnGlyProValAspLeuLysLysIle

                ACAAACTTTTTCAGAGGGGATCGTTGTAGAAGTCTAACTGGAAAACCCAAACTTTTCATT
481             ---------+---------+---------+---------+---------+---------+ 540
                TGTTTGAAAAAGTCTCCCCTAGCAACATCTTCAGATTGACCTTTTGGGTTTGAAAAGTAA
                ThrAsnPhePheArgGlyAspArgCysArgSerLeuThrGlyLysProLysLeuPheIle
```

FIG.4A

```
        ATTCAGGCCTGCCGTGGTACAGAACTGGACTGTGGCATTGAGACAGACAGTGGTGTTGAT
541     ---------+---------+---------+---------+---------+---------+  600
        TAAGTCCGGACGGCACCATGTCTTGACCTGACACCGTAACTCTGTCTGTCACCACAACTA
        IleGlnAlaCysArgGlyThrGluLeuAspCysGlyIleGluThrAspSerGlyValAsp
                  163

        GATGACATGGCGTGTCATAAAATACCAGTGGAGGCCGACTTCTTGTATGCATACTCCACA
601     ---------+---------+---------+---------+---------+---------+  660
        CTACTGTACCGCACAGTATTTTATGGTCACCTCCGGCTGAAGAACATACGTATGAGGTGT
        AspAspMetAlaCysHisLysIleProValGluAlaAspPheLeuTyrAlaTyrSerThr

        GCACCTGGTTATTATTCTTGGCGAAATTCAAAGGATGGCTCCTGGTTCATCCAGTCGCTT
661     ---------+---------+---------+---------+---------+---------+  720
        CGTGGACCAATAATAAGAACCGCTTTAAGTTTCCTACCGAGGACCAAGTAGGTCAGCGAA
        AlaProGlyTyrTyrSerTrpArgAsnSerLysAspGlySerTrpPheIleGlnSerLeu

        TGTGCCATGCTGAAACAGTATGCCGACAAGCTTGAATTTATGCACATTCTTACCCGGGTT
721     ---------+---------+---------+---------+---------+---------+  780
        ACACGGTACGACTTTGTCATACGGCTGTTCGAACTTAAATACGTGTAAGAATGGGCCCAA
        CysAlaMetLeuLysGlnTyrAlaAspLysLeuGluPheMetHisIleLeuThrArgVal

        AACCGAAAGGTGGCAACAGAATTTGAGTCCTTTTCCTTTGACGCTACTTTTCATGCAAAG
781     ---------+---------+---------+---------+---------+---------+  840
        TTGGCTTTCCACCGTTGTCTTAAACTCAGGAAAAGGAAACTGCGATGAAAAGTACGTTTC
        AsnArgLysValAlaThrGluPheGluSerPheSerPheAspAlaThrPheHisAlaLys

        AAACAGATTCCATGTATTGTTTCCATGCTCACAAAAGAACTCTATTTTTATCACTAAAGA
841     ---------+---------+---------+---------+---------+---------+  900
        TTTGTCTAAGGTACATAACAAAGGTACGAGTGTTTTCTTGAGATAAAAATAGTGATTTCT
        LysGlnIleProCysIleValSerMetLeuThrLysGluLeuTyrPheTyrHisEnd

        AATGGTTGGTTGGTGGTTTTTTTTAGTTTGTATGCCAAGTGAGAAGATGGTATATTTGGT
901     ---------+---------+---------+---------+---------+---------+  960
        TTACCAACCAACCACCAAAAAAAATCAAACATACGGTTCACTCTTCTACCATATAAACCA

        ACTGTATTTCCCTCTCATTTTGACCTACTCTCATGCTGCAG
961     ---------+---------+---------+---------+-  1001
        TGACATAAAGGGAGAGTAAAACTGGATGAGAGTACGACGTC
```

FIG.4B

INTERNATIONAL SEARCH REPORT

	International Application No PCT/CA 97/00824

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K5/08 G01N33/573

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	R L WANGE ET AL.: "F2(PMP)2-TAMzeta3, a novel competitive inhibitor of the binding of ZAP-70 to the T cell antigen receptor, blocks early cell signaling" JOURNAL OF BIOLOGICAL CHEMISTRY., vol. 370, no. 2, 13 January 1995, MD US, pages 944-948, XP002056490 see the whole document --- -/--	1,2

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of theinternational search	Date of mailing of the international search report
20 February 1998	06/03/1998

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

Ir ational Application No
PCT/CA 97/00824

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	S R EATON ET AL.: "Structure-activity relationship of peptides that block the association of PDGF beta-trceptor with phosphatidylinositol 3-kinase" PEPTIDES. CHEMISTRY, STRUCTURE AND BIOLOGY. PROCEEDINGS OF THE 14TH AMERICAN PEPTIDE SYMPOSIUM, JUNE 18-23, 1995, COLUMBUS, OHIO, USA, 1996, MAYFLOWER SCIENTIFIC LTD, ENGLAND, pages 414-415, XP002056491 see the whole document ---	1,2
A	M F GORDEEV ET AL.: "N-alpha-FMOC-4-phosphono (difluoromethyl)-L-phenylalanine: a new O-phosphotyrosine isosteric building block suitable for direct incorporation into peptides " TETRAHEDRON LETTERS., vol. 35, no. 41, 10 October 1994, OXFORD GB, pages 7585-7588, XP000616320 see the whole document ---	1,2
X,P	WO 97 08300 A (ARIAD PHARMACEUTICALS, INC.) 6 March 1997 see page 17, compound 9 -----	1,2

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Ir ational Application No
PCT/CA 97/00824

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9708300 A	06-03-97	AU 6960696 A	19-03-97

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